THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Evaluation of precursor and cofactor engineering strategies influencing fatty acid metabolism in *Saccharomyces cerevisiae*

ALEXANDRA BERGMAN

Department of Biology and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2019

Evaluation of precursor and cofactor engineering strategies influencing fatty acid metabolism in *Saccharomyces cerevisiae*

Alexandra Bergman
Gothenburg, Sweden 2019
ISBN 978-91-7597-868-0

Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 4549
ISSN 0346-718X

Department of Biology and Biological Engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone + 46 (0)31-772 1000

Cover: “Watercolor budding yeast”, illustrated by Elin Bergman.

Printed by Chalmers Reproservice
Gothenburg, Sweden 2019

Evaluation of precursor and cofactor engineering strategies influencing fatty acid metabolism in *Saccharomyces cerevisiae*

ALEXANDRA BERGMAN

Department of Biology and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Abstract

If humanity is to reduce the rate of climate change, it is essential that our societies switch to a more sustainable production of fuels and chemicals, which in turn depends on technological development. Oleochemical production via microbial catalysts – such as the yeast *Saccharomyces cerevisiae* – can use a considerably broader range of renewable substrates compared to the conventional production processes. Additionally, it enables optimization of the catalytical properties of the chosen host via metabolic engineering. Oleochemicals are derived from fatty acids (FAs), whose biosynthesis depends on the conversion of a substrate to cytosolic acetyl-CoA – the precursor for FA synthesis. FA synthesis additionally requires large amounts of the reducing cofactor NADPH. The primary aim of this thesis was to develop and evaluate metabolic engineering strategies with potential to positively influence FA production of *S. cerevisiae*, mediated via an increased supply of acetyl-CoA or NADPH.

A major portion of the thesis is focused around a heterologous metabolic pathway to produce acetyl-CoA based on the activity of a phosphoketolase (XFPK) and a phosphotransacetylase (PTA). This pathway theoretically allows to reduce carbon and energy losses compared to the native yeast system. We identified several efficient XFPK candidates with potential to generate a high flux through the pathway. Furthermore, we show that two endogenous proteins – Gpp1 and Gpp2 – efficiently degrade the XFPK-formed produced acetyl-phosphate (AcP) to acetate, accumulating during cultivation. We show that this limits the benefit of the heterologous pathway, likely due to increased proton decoupling and ATP consumption during acetate activation. When we co-expressed XFPK and PTA, deletion of *GPP1* appeared to be required to enable a significant flux towards acetyl-CoA during growth on glucose, reducing acetate accumulation. While a 25% increase in FA production was observed at the end of the glucose phase, the final titer was reduced by 20% compared to the control. We suggest that PTA expression negatively affects FA production during ethanol consumption due to low level of AcP during such conditions, leading to net flux from acetyl-CoA to AcP. Therefore, we propose that ethanol formation should be avoided in order to optimize XFPK/PTA use.

Regarding cofactor supply, we investigated if increasing activity of Stb5 – a transcriptional activator of genes involved in the pentose phosphate pathway (PPP) and NADPH production – could influence FA synthesis positively. *STB5* overexpression had a beneficial effect on FA production in the glucose phase, an effect shown to be independent of flux through the PPP. However, final titers were affected negatively, and transcriptomic analysis indicates that mechanisms were activated in cells to counteract a Stb5-imposed redox imbalance. This suggests that an effective drain of NADPH – e.g. during product formation – is required to prevent systemic negative effects of *STB5* overexpression.

The results produced within the scope of this thesis will serve as an aid in future metabolic engineering strategies targeting compounds relying on acetyl-CoA or NADPH.

Keywords: Sustainability, oleochemicals, fatty acids, *Saccharomyces cerevisiae*, acetyl-CoA, NADPH, metabolic engineering, phosphoketolase, transcription factor.

List of publications

This thesis is based on the work contained in the following publications (referred to as paper I-IV in the text):

I. **Bergman A**, Siewers V, Nielsen J, Chen Y. (2016) Functional expression and evaluation of heterologous phosphoketolases in *Saccharomyces cerevisiae*. *AMB Express*, **6**:115.

II. **Bergman A**, Hellgren J, Moritz T, Siewers V, Nielsen J, Chen Y. (2019) Heterologous phosphoketolase expression redirects flux towards acetate, perturbs sugar phosphate pools and increases respiratory demand in *Saccharomyces cerevisiae. Microbial Cell Factories,* **18**:25.

III. **Bergman A**, Vitay D, Hellgren J, Chen Y, Nielsen J, Siewers V. (2019) Effects of overexpression of *STB5* in *Saccharomyces cerevisiae* on fatty acid biosynthesis, physiology and transcriptome. *FEMS Yeast Research*, **19**:foz027.

IV. **Bergman A**, Wenning L, Siewers V, Nielsen J. (2018) Investigation of putative regulatory acetylation sites in Fas2p of *Saccharomyces cerevisiae*. Manuscript, available at bioRxiv (https://doi.org/10.1101/430918).

Additional publications not included in this thesis:

V. **Bergman A** and Siewers V**.** (2016) Biofuels for aviation; feedstocks, technology and implementation, Chapter 7 – "Metabolic Engineering Strategies to Convert Carbohydrates to Aviation Range Hydrocarbons". Editor: Christopher Chuck. 1st ed. 2016, London: Elsevier.

Contribution summary

I. Contributed to the design of the study, performed all experiments, analyzed the data, and wrote the paper.
II. Designed the study, performed most experiments, analyzed most of the data, and wrote the paper.
III. Designed the study, performed or supervised all experiments, analyzed most of the data and wrote the paper.
IV. Designed the study, performed most experiments, analyzed most of the data, and wrote the paper.

V. Carried out the literature review and wrote part of the chapter.

Preface

This dissertation is submitted for the partial fulfillment of the degree of Doctor of Philosophy. It is based on work carried out between April 2013 and August 2018 in the Systems and Synthetic Biology group, Department of Biology and Biological Engineering, Chalmers University of Technology, under the supervision of Professor Jens Nielsen, Dr. Verena Siewers and Dr. Yun Chen. The research was funded by the Knut and Alice Wallenberg Foundation, the Novo Nordisk Foundation, the Swedish Foundation for Strategic Research, The Swedish Research Council, FORMAS, and Carl Tryggers Foundation. Personal funding has been received from Ångpanneföreningens Research Foundation and the Chalmers Research Foundation.

Alexandra Bergman, May 2019

Table of contents

To Ingrid

1 Introduction

1.1 Oleochemicals - from a global to a cellular perspective

A global perspective

When I initiated writing this thesis in Gothenburg 2018, my attention was effectively brought to the topic of climate change, as the high temperatures and lack of rain observed in Sweden during May to July were extreme. As of 2017, the global temperature has increased by 0.9°C relative to the average 1951-1980 temperature, and across the globe extreme weather conditions are notably more common. Climate change is an inevitable result of rising levels of greenhouse gas (GHG) emissions, of which CO_2 already by 1950 had surpassed the highest recorded level during the last 420.000 years [1] – approximately 300 ppm – and in 2016 crossed the 400 ppm mark (https://climate.nasa.gov/vital-signs/carbon-dioxide/). Therefore, a rapid reduction of GHG emissions is imperative.

In order to create a sustainable society with reduced GHG footprint, the global economy must undergo a major transformation. The prevailing economic model can be described as linear – it is highly dependent on manufacturing processes where non-renewable resources such as fossil fuels are used as raw material and our waste is largely dumped or burnt. A paradigm shift would be to fully move into a circular economy, which seeks to reduce resource consumption and maximize recycling of waste-streams [2]. Consumption will inevitably continue, which is why production routes must shift toward sustainable operation. The so-called bioeconomy describes a system in which "the basic building blocks for materials chemicals and energy are derived from renewable biological resources" [3].

Oleochemicals – applications and production

A product group that today exclusively relies on biomass for production is oleochemicals. Oleochemicals are aliphatic molecules that are used to produce a diverse range of products, from consumer goods such as cosmetics, shampoo and detergents to industrial lubricants, emulsifiers and transportation fuels [4]. The common precursor to build such chemicals are fatty acids (FAs), which are in abundance in biological lipids, such as vegetable oils and animal fat. As an illustration of the applicability and demand for FAs, the annual growth of the world-wide vegetable oil market was a remarkable 4.5% during the last decade (2008-2017), largely

due to a growing biofuel and oleochemical production [5]. The growth is predicted to be closer to 2% over the next decade, but vegetable oils will still represent one of the fastest growing commodities. Even if the raw material required for production of oleochemicals and biofuels technically is renewable, vegetable oil production is considered environmentally unsustainable in the short-term perspective. This is due to the extensive time required to repay the high "carbon debt" associated with the establishment of oil crops such as palm and soy in areas which currently hold rainforests. As an example, it is estimated that the time to repay the carbon debt from palm biodiesel produced from crops established in Indonesian or Malaysian peatland rainforests is 423 years [6]. During this time, the net emission of GHGs from using such biofuels exceed that of the fossil-based fuels they replace. For example, the expanse of efficient palm oil crops causes deforestation in tropical regions, which by itself leads to a vast increase in GHG emissions, biodiversity loss and soil degradation over time [7].

The microbial catalyst and fatty acid biosynthesis

A way to limit the requirement of oil crops in oleochemical production, and thereby reduce environmental impact, is to rely on microbial catalysts. Fermentation-based oleochemical production offers a great advantage over the traditional chemical conversion of vegetable oils, as a broad range of plant substrates potentially can be utilized. For example, the carbohydrates in lignocellulosic material such as wood and grass represent an abundant substrate stream, and these type of plants can be cultivated in various climate types [8]. A microbial catalyst can be precisely engineered to catalyze multiple steps in a bioconversion process, as traits and enzymatic capabilities from different species can be transferred into a representative host. For example, lignocellulose degradation observed by fungal species as well as the biosynthesis of alkanes observed by certain cyanobacteria can theoretically be combined into a single host.

In this context, the yeast *Saccharomyces cerevisiae* has for long been a model organism for the eukaryote cell, resulting in both detailed information about its biology and an arsenal of tools to manipulate its genome being available. Furthermore, it is a robust organism tolerating harsh cultivation conditions such as low pH and it is resistant to phage-contamination, which commonly affects bacterial hosts. Consequently, *S. cerevisiae* is a commonly observed microbial host for bioconversion processes, such as the large-scale production of succinic acid by BioAmber, isobutanol by GEVO and farnesense by Amyris [9]. During the last five years, *S. cerevisiae* has been intensely explored and developed as catalyst for oleochemical production [10], such as free FAs [11-16], fatty alcohols [13, 14, 17], phospholipids [16], triacylglycerols [18], long/short chain FA species [19-22], alkanes/alkenes [14, 23-25], specialty lipids [26-28] FA ethyl esters [13, 29-33] and wax esters [34, 35].

In brief, oleochemical production in *S. cerevisiae* is dependent on the degradation of substrate molecules to the central metabolite acetyl-CoA. Acetyl-CoA is a thioester comprised of a molecule of acetic acid bound to the thiol "coenzyme A", which is utilized as an acyl-group carrier within the cell [36]. The formed acetyl-CoA can subsequently be used as precursor in the resource-intensive synthesis of FAs. The production of one molecule of octadecanoic acid – a FA comprising 18 carbon molecules – requires consumption of 9 acetyl-CoA, 8 molecules of the molecular energy equivalent ATP and 16 molecules of the reducing cofactor NADPH. The enzymes responsible for FA synthesis are acetyl-CoA carboxylase (Acc1), converting acetyl-CoA to malonyl-CoA [37] and the fatty acid synthase (FAS): a large enzyme complex comprised of two separate proteins, Fas1 (β) and Fas2 (α), which each has multiple catalytic domains. The FAS utilizes acetyl-CoA as a priming unit and malonyl-CoA as an extender unit during FA elongation [38]. FA synthesis is a highly controlled cellular process and has been shown to be regulated at the transcriptional level [39], via post-translational phosphorylation [40, 41] as well as feed-back inhibition mediated by acyl-CoA [42].

1.2 Objectives

As previously mentioned, plenty of resources have been invested into the area of developing *S. cerevisiae* as a host for oleochemical production. Nevertheless, the intricacy of biological systems offers numerous alternative solutions with the ability to improve a particular aspect of a process. Moreover, even though the process of FA synthesis is highly researched in *S. cerevisiae*, there are aspects which remain unstudied. The research laying the ground for this doctoral thesis has focused on three separate aspects with potential to positively influence FA synthesis and/or to generate a better understanding of the FA synthesis process: 1) precursor supply of acetyl-CoA, 2) cofactor supply of NADPH, and 3) enzyme regulation. A short background and a description of the objective of these areas of study are presented below.

Precursor supply: Acetyl-CoA

The native route for cytosolic acetyl-CoA supply in *S. cerevisiae* is carbon- as well as energy-inefficient, limiting the theoretical yield of any acetyl-CoA derived product. As high product-on-substrate yield is one of the most important factors for a successful biorefinery, there has been a high research focus on shifting to alternative, heterologous routes for cytosolic acetyl-CoA supply. The route that theoretically is among the most promising in terms of optimal yield is based on a coordinated expression of a phosphoketolase (XFPK) and a phosphotransacetylase (PTA), as the route bypasses both carbon and energy loss [43]. Previous research, utilizing a XFPK from *Aspergillus nidulans* in *S. cerevisiae* in order to enhance acetyl-CoA derived product formation, presented conflicting results with regards to the efficacy of the introduced heterologous route [32, 44, 45]. Efficient enzyme catalysis is essential if carbon fluxes should be successfully diverted within the cell. In **Paper I** we screened several heterologous phosphoketolase candidates for functional expression in *S. cerevisiae* and high catalytic efficiency. This enabled us to identify several efficient enzyme candidates, whose expression however had a negative impact on yeast growth. A growth defect represents a significant drawback in biorefinery applications, as it is likely to reduce both yield and productivity of a process. For this reason we characterized effects of expression of the most efficient XFPK identified in Paper I from *Bifidobacterium breve* (Xfpk(BB)), results presented in **Paper II**. Ultimately, we evaluated how co-expression of XFPK and PTA species affected growth and/or FA production in different yeast strains, the results of which are enclosed and discussed within the thesis (**Chapter 3.4**).

Cofactor supply: NADPH

NADPH is the abbreviation for the reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$), which is utilized by cells as a reducing agent in biosynthetic reactions. NADPH biogenesis, and in particular flux through the oxidative pentose phosphate pathway (PPP), is required to be a highly regulated process due to the global effect the redox state has on cellular metabolism. NADPH supply is considered to be increased in response to a higher demand, for example observed under levels of oxidative stress and an increased biosynthetic requirement [46]. In a reversed approach we evaluated if it was possible to enhance FA biosynthesis, a process that requires large amounts of NADPH, by forcing the cell to increase its NADPH supply. The transcription factor (TF) Stb5 has been shown to be an activator of multiple genes related to the PPP and NADPH production in response to oxidative stress [47]. In **Paper III** we evaluated if overexpression of *STB5* could be utilized as a metabolic engineering strategy to positively influence FA synthesis. In the same paper, we studied what effects overexpression had on physiology as well as global transcription.

Enzyme regulation: FAS

Lysine acetylation is a common post-translational modification which has emerged as an important regulator of many metabolic enzymes [48]. Henriksen et al. conducted an acetylome study of *S. cerevisiae*, identifying more that 4000 unique acetylation sites on proteins [49]. Yet, there have been few reports validating the potential regulatory function of acetylation sites in *S. cerevisiae*. Acetyl-CoA acts as the donor for protein acetylation, and the intracellular acetyl-CoA levels have been shown to correlate with the degree of protein acetylation [50]. Among the enzymes found to be acetylated by Henriksen et al, Acc1 and Fas1/Fas2 were reported to have multiple acetylation sites. As these enzymes are located at the branchpoint of metabolism of acetyl-CoA, the donor for protein acetylation, it is possible that protein acetylation could be a regulatory determinant of function. This has been shown to be the case for the human fatty acid synthase [51]. The identification of post-translational regulatory sites on enzymes poses an opportunity to deregulate connected cellular processes. With the overall aim to enhance FA synthesis in *S. cerevisiae* in mind, in **Paper IV,** three acetylation sites in Fas2 were targeted for investigation for a putative regulatory role in FA synthesis.

1.3 Thesis structure

Chapter 2 is dedicated to the presentation of relevant background to the topics of this thesis. It covers important features of *S. cerevisiae* metabolism, such as an overview of the central carbon metabolism and important physiological responses. Furthermore, due to the central role of acetyl-CoA, FA synthesis and redox metabolism in this thesis, aspects such as biosynthetic pathways and regulation of these processes are discussed in greater detail. The last subchapter gives an overview of the cell factory concept and metabolic engineering principles and methodology.

The three following chapters are initiated with an introduction of previous work with relevance for the undertaken subproject, and proceed to present the results and connected discussion of the studies conducted within the scope of the chapter. **Chapter 3** covers the results of **Paper I-II**, in which heterologous enzymes with ability to influence acetyl-CoA supply were evaluated for functionality and impact of expression on *S. cerevisiae* physiology. The last subchapter (**Chapter 3.4**) also contains results which are not part of a manuscript. **Chapter 4** presents results of **Paper III** in which the effect of overexpression of the transcription factor Stb5 was evaluated. Lastly, **Chapter 5** presents the study where the potential regulatory function of acetylation sites in Fas2 was evaluated, covering **Paper IV**.

Chapter 6 concludes the thesis presenting key conclusions and future perspectives of each individual project described within this thesis.

2 Background

Saccharomyces cerevisiae, baker's yeast, has been a key player in the development of the scientific fields of microbiology, biochemistry and genetics. Long before this scientific revolution in biology, yeast constituted a "kitchen-aid" for thousands of years for bread baking, wine making and beer brewing. This is due to its excellent ability to break down sugar molecules while producing ethanol and carbon dioxide – the intoxicating and bread-lifting agents of brewed liquids and bread, respectively.

The first conceptional understanding of the chemical process of fermentation was made by Antoine Lavoisier in 1789, who proposed that sugar was converted into alcohol and carbon dioxide during wine fermentation [52]. Yet, it was not until the 1836-1837 that it was recognized that organisms were responsible for this conversion process, independently shown by Charles Cagniard-Latour, Friedrich Kützing and Theodor Schwann [52]. Louis Pasteur studied the physiology of yeasts in the late 1850s, established their role in fermentation, and determined the differences in how sugar was processed by the yeasts during aerobic and anaerobic conditions [53]. The first pure yeast cultures were isolated in 1883 by Emil Christian Hansen, at the time employed by the Carlsberg Laboratory in Copenhagen, thereby initiating a revolution in the brewing industry as consistent flavor and quality of the products could be obtained [54].

This basic metabolic trait of yeast is still the foundation for bread and alcohol fermentations in industry. Nevertheless, the accomplishments in microbiology, biochemistry and genetics have propelled the birth of scientific disciplines which allow to engineer biological systems. This has helped to optimize yeast as an industrial organism, not only for the "task" millions of years of evolution led it to execute, but also for completely new purposes. In the field of metabolic engineering, genetic traits are manipulated or introduced into a host organism with the aim to influence its metabolism - the networks of biochemical reactions occurring within cells. This has enabled the creation of "cell factories", which can efficiently convert simple raw materials such as sugars into complex chemicals.

In order to improve the interpretations of the topics of this thesis, the following subchapters present the basis of central carbon metabolism in yeast and specific metabolic branches with special connection to the projects of this thesis. Lastly, the concept of the cell factory and metabolic engineering principles will be presented.

2.1 Central carbon metabolism of *Saccharomyces cerevisiae*

Glycolysis

A hexose sugar molecule is within the cell primarily transformed through the process of glycolysis – a coordinated action of numerous enzymes with specificity for the particular intermediary metabolites, an overview of which is found in **Figure 1**. The elucidation of the main reactions of glycolysis was made possible after Eduard Buchner in 1897 showed that a cell-free extract of yeast could still ferment sugars. It was thus shown that glycolysis was a process not directly dependent on living cells but rather chemical catalysis made possible by proteins [54]. Glycolysis was recognized as a general biochemical pathway, and the details of the pathway were studied and fully described both in yeast and muscle cells by the first half of the twentieth century [55].

Altough *S. cerevisiae* may consume different types of hexose sugars, the preferred substrate is glucose – the dominant carbohydrate in plant biomass – which can be readily fermented at high rates even under anaerobic conditions [56]. The two glucose-isomers mannose and fructose as well as the hexose galactose are fermentable carbon sources for *S. cerevisiae*. However, while the two former are readily fermented, yeast growth rate on galactose is about a third of that observed for growth on glucose and mainly of respiratory character [57]. Hexose sugars are imported by facilitated transport via a family of hexose transporters (HXT). These differ with respect to substrate specificity, affinity for glucose and transcriptional and posttranscriptional regulation [58, 59].

Once imported into the cell, glucose as well as mannose and fructose are first phosphorylated by the action of hexokinases (encoded by *HXK1* and *HXK2*) in an ATP dependent process, yielding glucose-6-phosphate (G6P), mannose-6-phosphate (M6P) and fructose-6-phosphate (F6P), respectively. Galactose is converted to G6P through the so-called "Leloir pathway", via formation of galactose-1-phosphate and glucose-1-phosphate. G6P and M6P are converted into F6P by glucose-6-phosphate isomerase (encoded by *PGI1*) and phosphomannose isomerase (encoded by *PMI40*), respectively. Another molecule of ATP is consumed when F6P is converted into fructose-1,6-bisphosphate (F1,6BP) by 6-phosphofructo-1-kinase (encoded by *PFK1* and *PFK2*). F1,6BP is thereafter cleaved into two three-carbon compounds: glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by fructose-1,6-bisphosphate aldolase (encoded by *FBA1*). Triose phosphate isomerase (encoded by *TPI1*) catalyzes the interconversion of DHAP and GAP, completing the so-called "investment-phase" of glycolysis with formation of two molecules of GAP.

The "energy generation phase" is initiated by the conversion of GAP to 1,3-bisphosphoglycerate (1,3BPG) under NADH-formation through the action of glyceraldehyde-3-phosphate dehydrogenase (encoded by *TDH1*, *TDH2* and *TDH3*), commonly referred to as GAPDH. One molecule of ATP is subsequently released when 1,3BPG is converted into 3-phosphoglycerate (3PG) via phosphoglycerate kinase (encoded by *PGK1*). Phosphoglycerate mutase (encoded by *GPM1*) coverts 3PG into 2-phosphoglycerate (2PG). Phosphopyruvate hydratase, also known as enolase (encoded by *ENO1* and *ENO2*), subsequently converts 2PG into phosphoenolpyruvate (PEP). In a final step, pyruvate kinase (encoded by *PYK1* (*CDC19*) and *PYK2*) catalyzes the conversion of PEP to pyruvate while producing an additional molecule of ATP. In summary, the enzymatic processing of one molecule of glucose results in two molecules each of pyruvate, NADH and ATP, without any requirement of molecular oxygen.

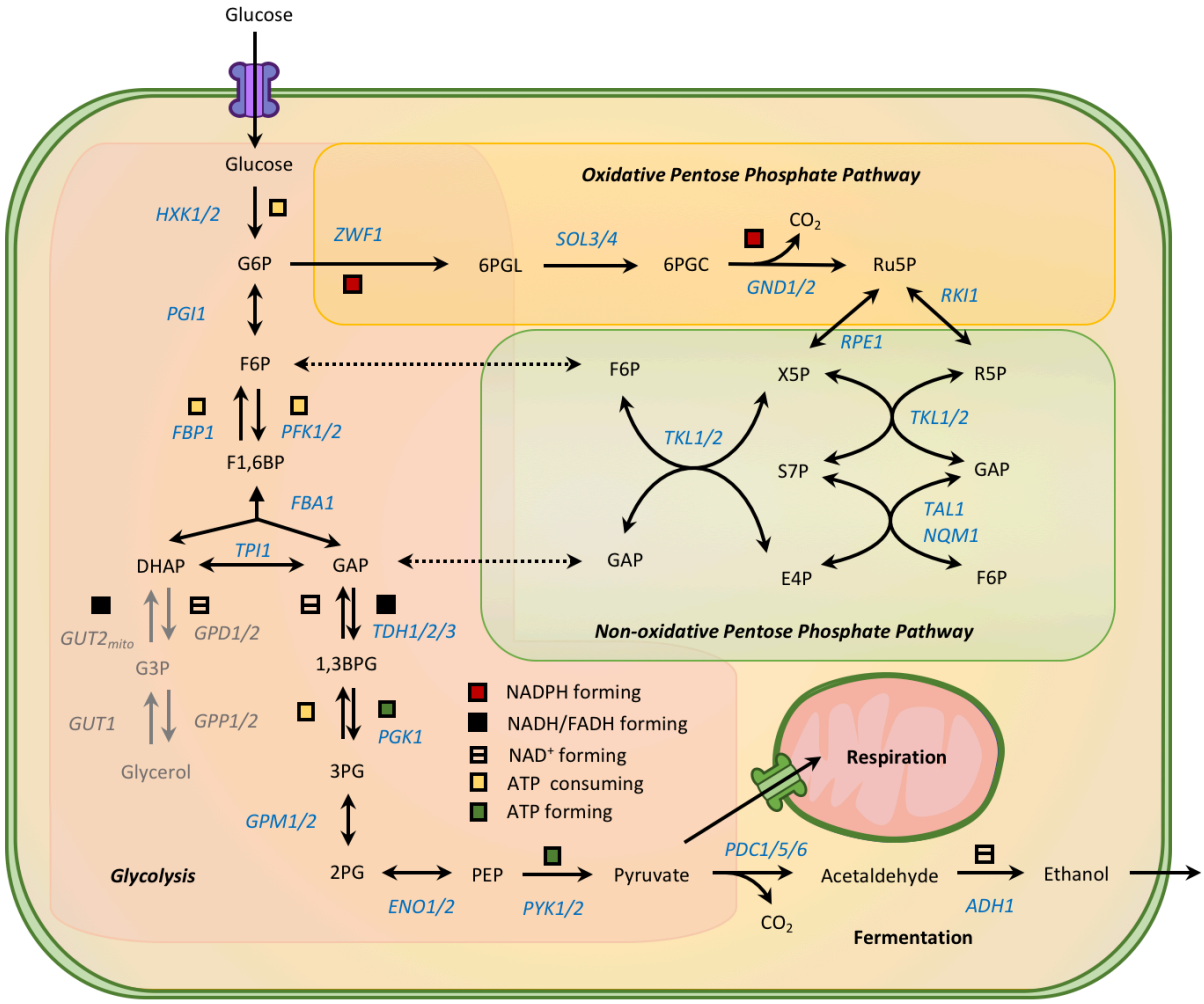


Figure 1. Central carbon metabolism: glycolysis (pink box), the oxidative (yellow box) and non-oxidative (green box) part of the PPP, and the pyruvate branchpoint of respiratory or fermentative growth (lower right corner). Glycerol synthesis/utilization pathway is also shown in the "Glycolysis box", indicated in grey.

The pentose phosphate pathway

As shown in **Figure 1**, an important route diverting carbon from glycolysis is the pentose phosphate pathway (PPP). The pathway generates NADPH and precursors for nucleotide and amino acid synthesis, the sugars ribose-5-phosphate (R5P) and erythrose-4-phosphate (E4P), respectively. The PPP has two distinct branches: an oxidative branch that comprises three enzymatic reactions of which two produce NADPH, and a non-oxidative branch that comprises four reactions and rearranges the carbon molecules of the sugar phosphates into C_3-C_7 compounds.

The first step of the oxidative branch of the PPP is catalyzed by glucose-6-phosphate dehydrogenase, encoded by *ZWF1*, and produces 6-phosphogluconolactone (6PGL) and one molecule of NADPH when G6P is oxidized. Subsequently, 6-phosphogluconolactonase, encoded by *SOL3* and *SOL4*, acts upon 6PGL to produce 6-phosphogluconate (6PGC). The last step of the oxidative PPP is catalyzed by 6-phosphogluconate dehydrogenase, encoded by *GND1* and *GND2*, that oxidizes 6PGC further into ribulose-5-phoshate (Ru5P) and CO_2 resulting in an additional molecule of NADPH. The enzymatic reactions of the oxidative PPP are largely considered unidirectional, and the regulation of flux into the pathway is reviewed in more detail in section 2.4, describing NADPH synthesis routes in greater detail.

The non-oxidative part of the PPP, on the other hand, is comprised of reversible interconversions of C_3-C_7 sugar phosphates connecting intermediates of the PPP with those of glycolysis. The Ru5P formed from the oxidative PPP can either be converted by ribose-5-phosphate ketol-isomerase (encoded by *RKI1*) to R5P or ribulose-5-phosphate 3-epimerase (encoded by *RPE1*) to xylulose-5-phosphate (X5P). The two pentose sugars R5P and X5P can be recombined into one C7 and one C3 sugar phosphate, namely seduheptulose-7-phosphate (S7P) and the glycolytic intermediary GAP. The reaction is catalyzed by a transketolase (encoded by *TKL1* and *TKL2*), activity of which is dependent on the cofactor thiamine diphosphate (ThPP) [60]. A transaldolase (encoded by *TAL1* and *NQM1*) can further rearrange S7P and GAP into F6P and E4P, which can be degraded in glycolysis or utilized in aromatic amino acid and vitamin B synthesis, respectively. Transketolases furthermore catalyze the interconversion of E4P and X5P into F6P and GAP.

A majority of the above described enzymes of the canonical PPP were described in the first half of the 20th century, and a draft version of the complete pathway was presented in 1955 [61]. However, as late as in 2011, the PPP enzyme sedoheptulose-1,7-bisphosphatase (encoded by *SHB17*) was characterized in yeast. It catalyzes a non-reversible formation of S7P from sedoheptulose-1,7-bisphosphate (SBP), that in turn is formed via interconversion of DHAP and E4P catalyzed by the glycolytic aldolase Fba1 [62]. Thus, Shb17 constitutes an additional link between glycolysis and the PPP (not shown in Figure 1).

Fate of pyruvate and the Crabtree effect

The pyruvate produced from glycolysis has different destinations within the yeast cell. The molecule is located at a branch-point of dissimilatory and assimilatory metabolic pathways and simultaneously between respiratory and fermentative metabolism [63], illustrated in **Figure 1**. If destined for respiratory assimilation, pyruvate is imported into the mitochondria and subsequently oxidized within the tricarboxylic acid (TCA) cycle to produce CO_2 along with reducing equivalents for the respiratory chain. Furthermore, pyruvate can be carboxylated via pyruvate carboxylase (encoded by *PYC1*) into oxaloacetate in an ATP-dependent anaplerotic reaction. Conversely, during fermentative growth, the majority of the pyruvate molecules are decarboxylated by cytosolic pyruvate decarboxylase (encoded by *PDC1*, *PDC5* and *PDC6*) to acetaldehyde and CO_2. The acetaldehyde is then reduced to ethanol in a reaction primarily catalyzed by alcohol dehydrogenase 1 (encoded by *ADH1*) [64].

Respiratory dissimilation of glucose via the mitochondrial pathways is more energetically favorable in terms of ATP yield compared to fermentative conversion of glucose into ethanol. In *S. cerevisiae*, pure fermentation results in 2 ATP per consumed molecule of glucose without any consumption of oxygen, while it is estimated that 18 ATP can be formed through respiration [65]. The additional ATP formed via respiration stem from oxidative phosphorylation when electrons are donated from NADH to ultimately form molecular oxygen in the mitochondrial electron transport chain. During fermentative growth and ethanol formation, acetaldehyde is instead utilized as an electron acceptor of NADH without any concurrent ATP production. The reductive formation of ethanol allows the yeast cell to grow while maintaining redox balance in conjunction with NADH-producing glycolysis [66].

In contrast to facultative anaerobic species, which exclusively perform fermentation if oxygen is lacking, *S. cerevisiae* diverts a majority of provided glucose towards ethanol formation even in the presence of oxygen if glucose is present in excess – a phenomenon referred to as the Crabtree effect [67]. Two distinct effects have been observed, one "short-term" and one of "long-term" character. The short-term effect refers to the immediate onset of alcoholic fermentation after addition of an excess of sugar to a sugar-limited culture [63]. The effect is believed to represent a saturation of respiratory metabolism, and is commonly referred

to as "overflow metabolism". Exposure to elevated glucose levels triggers a transcriptional downregulation of a majority of genes encoding enzymes of the TCA cycle and respiratory chain [68]. Such repression effectively blocks respiratory routes for pyruvate assimilation, and is believed to be the cause of "long-term" Crabtree effect, occurring at high growth rates [69].

The Crabtree effects is counterintuitive if solely considering the theoretical ATP yield as a determining factor for mode of growth. However, an advantage emerges if considering that ATP production can be performed at a higher rate by using aerobic fermentation, permitting a higher growth rate. This phenomenon is clearly demonstrated by the lower growth rate of *S. cerevisiae* on non-fermentable carbon sources such as ethanol [70]. A higher growth rate would provide a selective advantage in settings when there is a competition for limited resources, referred to as the rate/yield trade-off (RYT) hypothesis, described by Pfeiffer et al. [71]. Furthermore, by constraining a stoichiometric model by the allocation of protein mass, Nilsson and Nielsen recently showed that aerobic fermentation is more catalytically efficient than respiration, i.e. more ATP can be produced per unit of protein [72].

An alternative hypothesis suggests that the Crabtree effect evolved in yeast as a mechanism to defend sugar rich resources, as the produced ethanol is antiseptic and limits growth of sensitive species, a theory termed the "make-accumulate-consume" (MAC) strategy [73]. In favor of this, the resurrection of the last common ancestor of the ethanol-forming dehydrogenase Adh1 and the ethanol-consuming Adh2 kinetically resembles Adh1 [74]. Thus, ancestral yeast was highly unlikely to perform aerobic fermentation as produced ethanol could not be consumed afterwards. Furthermore, the Adh-duplication event is estimated to coincide with 1) the time point of the duplication of other genes involved in controlling flux from hexose to ethanol (e.g. PDC) and 2) the Cretaceous age, during which an excess of fruits and fermentable sugars became available [74].

As aerobic low-yield metabolism exists also in other biological systems, such as bacterial and human systems [75], it has been argued that RYT likely is a more influential factor compared to MAC [76]. More recently, de Alteriis et al. argued that the Crabtree effect, as well as the corresponding Warburg effect commonly observed in cancer cells, could have evolved as a protection against the phenomenon "sugar-induced cell death" (SCID) and ATP-depletion [77]. It has also been suggested that an upper limit of energy dissipation is the trigger of the Crabtree/Warburg effect [78], which the authors hypothesize is a type of 'safety valve' to prevent cells from damage. Thus, the evolutionary background to the Crabtree effect is still under discussion.

2.2 Acetyl-CoA biosynthesis and cellular utilization

Acetyl-CoA is a key intermediate in central carbon metabolism. It acts as fuel in the mitochondrial TCA cycle, a process that in part generates energy for the cell, in part produces essential precursors for amino acid and nucleotide synthesis. Acetyl-CoA also represent the precursor for fatty acid and isoprenoid synthesis localized to the cytosol and it is the degradation product of β-oxidation, i.e. fatty acid degradation, in the peroxisomes [79]. Furthermore, acetyl-CoA acts as the acetyl-donor in protein acetylation, a post-translational event occurring in mitochondria, nucleus as well as cytosol [50]. Clearly, this multifaced metabolite is of particular importance in cellular metabolism, and several aspects of its biosynthesis and metabolism are presented in this section. An overview of *S. cerevisiae* acetyl-CoA and C_2 carbon metabolism is shown in **Figure 2**.

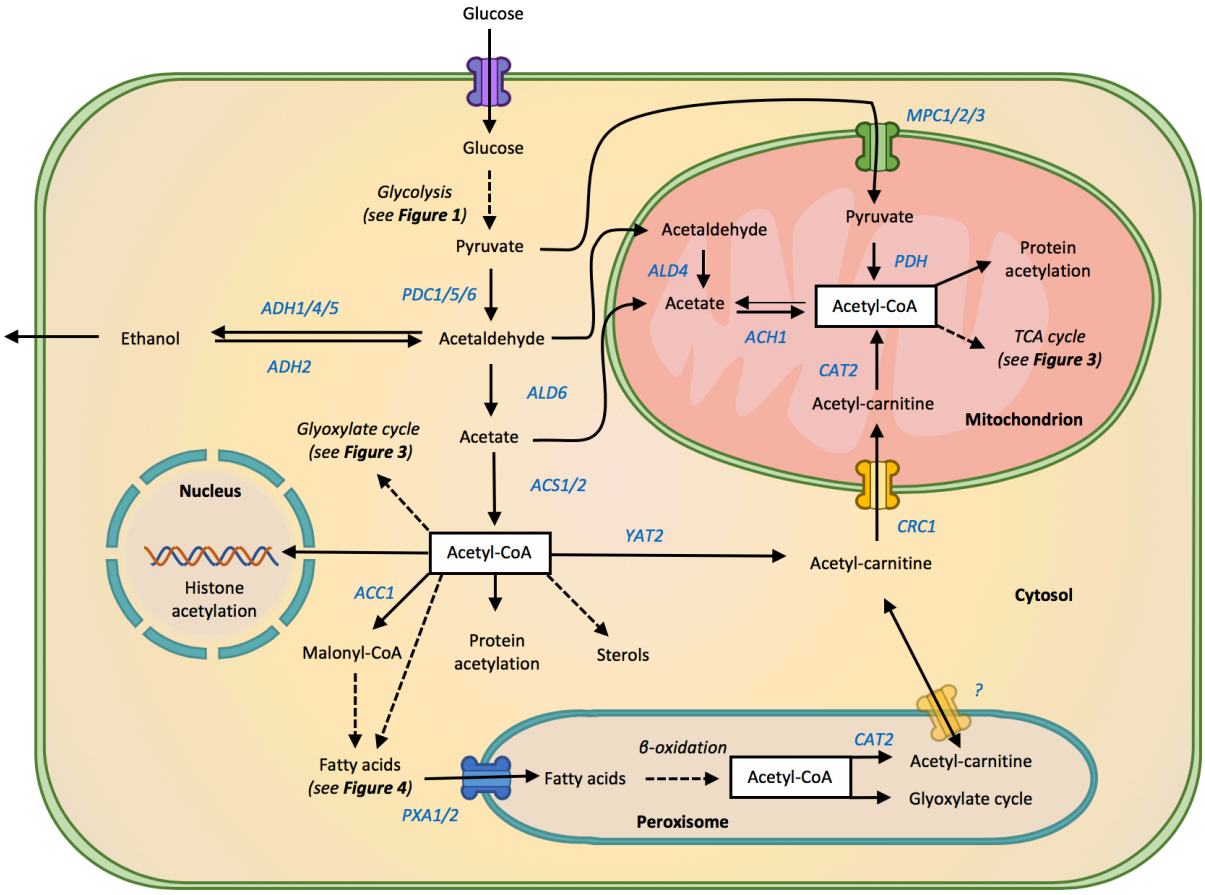


Figure 2. Acetyl-CoA synthesis, transport and utilization in *S. cerevisiae*. The two major routes for acetyl-CoA synthesis in yeast are 1) via the PDH-complex in the mitochondria, and 2) the "PDH-bypass" in the cytosol, comprising the activity of Pdc/Ald/Acs-enzymes. Acetyl-CoA can also be formed via degradation of fatty acids in the peroxisomes and through acetate activation by Ach1. Adapted from Galdieri et al. [80].

Acetyl-CoA biosynthesis

There are two major routes for acetyl-CoA synthesis in *S. cerevisiae* separated into different subcellular compartments: the mitochondria and the cytosol.

In the mitochondria, pyruvate is first required to be imported from the cytosol via the mitochondrial pyruvate carriers (encoded by Mpc1, Mpc2 and Mpc3). The functional carrier is reported to be a complex of primarily Mpc1/Mpc2 in respiratory conditions and Mpc1/Mpc3 in fermentative conditions [81]. Acetyl-CoA is then directly synthesized from pyruvate in an irreversible oxidative decarboxylation catalyzed by the pyruvate dehydrogenase (PDH) complex. The complex is composed of three catalytic subunits encoded by five structural genes (*PDA1*, *PDB1*, *LAT1*, *LPD1* and *PDX1*), and requires participation of five coenzymes (FAD, NAD^+, ThPP, CoA and lipoic acid) to function [63]. The PDH complex is regulated on multiple levels, including transcriptional control of *LPD1* expression via glucose catabolite repression and Hap2/3/4-mediated activation, and post-translational inactivation of the complex by phosphorylation [82, 83]. The formed acetyl-CoA is fed into the TCA cycle via a condensation reaction with oxaloacetate, catalyzed by the mitochondrial isoforms of citrate synthase (encoded by *CIT1* and *CIT3*) [84].

In the cytosol, acetyl-CoA is formed from pyruvate in a three-step process, commonly referred to as the "PDH bypass" [63]. It is initiated with the decarboxylation of pyruvate to acetaldehyde catalyzed by the Pdc enzymes. The two last enzymatic steps coincide with utilization of ethanol (see **Figure 2**). After glucose is consumed during aerobic fermentation,

S. cerevisiae undergoes through a diauxic shift, a process during which the growth temporarily is ceased while the cellular protein composition is remodeled to match respiratory metabolism. This involves a de-repression of genes encoding enzymes required for ethanol consumption, the glyoxylate cycle, gluconeogenesis and mitochondrial function [85, 86]. The non-fermentable carbon sources produced during the exponential phase (ethanol, glycerol and acetate) can thereafter be consumed, albeit at a lower growth rate compared to exponential growth on glucose [70]. When growing on ethanol, cytosolic acetyl-CoA formation is initiated by the oxidation of ethanol to acetaldehyde while NAD^+ simultaneously is reduced to NADH, a reaction mainly catalyzed by alcohol dehydrogenase 2 (encoded by *ADH2*) [64, 87]. Acetaldehyde is thereafter oxidized further to acetate. There are three cytosolic acetaldehyde dehydrogenases in *S. cerevisiae*; Ald2, Ald3 and Ald6. Of these, the NADPH-dependent Ald6 is responsible for the vast majority of the acetate formation [88]. Ald2 and Ald3 are instead involved in β-alanine synthesis – an intermediate in pantothenic acid and coenzyme A synthesis (discussed in more detail later in this subchapter)[89].

Cytosolic acetyl-CoA is formed from acetate via the action of acetyl-CoA synthetase (encoded by *ACS1* and *ACS2*). Expression of *ACS1* is subjected to glucose repression while *ACS2* is constitutively expressed, hence, a deletion mutant of *ACS2* fails to grow in high concentration of glucose [90]. Acs1 is believed to have a shared distribution between the cytosol and peroxisome, while Acs2 likely resides in the cytosol, the nucleus, and possibly in the endoplasmic reticulum (ER) [91-94]. Two equivalents of ATP are required for the synthesis of cytosolic acetyl-CoA, as ATP is hydrolyzed to AMP [43]. This processes reduces the respiratory ATP yield of ethanol and acetate compared to direct consumption of pyruvate via the TCA cycle.

An alternative way for acetyl-CoA formation relates to diffusion of undissociated acetate – produced via the PDH bypass – over the mitochondrial membrane. The gene *ACH1* encodes as a mitochondrial enzyme, which first was considered to be a acetyl-CoA hydrolase [95, 96], but later was found to have a much greater preference for transferring CoA from various acyl-CoA species to organic acids, such as acetate [97]. While the reaction is not considered influential under normal circumstances, it has been shown to be important for growth on acetate and if alternative routes for mitochondrial acetyl-CoA supply are inactivated [97, 98]. Furthermore, the less pronounced acetyl-CoA hydrolase catalytic properties of Ach1 have been shown to be essential for growth in a strain lacking pyruvate decarboxylase [99].

Lastly, acetyl-CoA formation is also dependent on biosynthesis of CoA, which is not consumed but instead recycled once an acetyl- and other acyl-groups are shuttled forward in metabolic pathways. CoA is a thiol which in *S. cerevisiae* can be synthesized *de novo* in a 13-step pathway, starting from pyruvate and valine, additionally requiring ATP, β-alanine and cysteine as substrates throughout the synthesis process [100, 101]. Alternatively, the intermediate pantothenate (vitamin B_5) can be imported via the high-affinity transporter Fen2 [102], leaving five reactions to be catalyzed (encoded by *CAB1-CAB5*) in a metabolic pathway that is believed to be universal among organisms [103]. Cab1 catalyzes the step that is considered to be rate limiting in CoA-biosynthesis, in which pantothenate is phosphorylated while consuming ATP, and the enzyme is strongly inhibited by acetyl-CoA [104]. Subsequently, a heterometric protein complex consisting of Cab2-Cab5 and the nonessential proteins Sis2 and Vhs3, which bear resemblance to Cab3, catalyze the remaining steps of CoA formation [105].

Acetyl-CoA utilization and the TCA/glyoxylate cycles

In contrast to metabolites such as ethanol, acetaldehyde and acetate, acetyl-CoA formed in the cytosol cannot travel freely between different subcellular organelles by means of diffusion. However, acetyl-CoA can readily move back and forth between the cytosol and the nucleus, referred to as the nucleocytosolic pool, which is biochemically separated from the mitochondrial pool [94]. The acetyl-CoA can be linked to carnitine by the catalytic action of carnitine acetyltransferases (CATs) [106], and thereafter transported to the mitochondrial compartment via the transporter protein Crc1 [107], shown in **Figure 2**. There are three known CATs in *S. cerevisiae*: Cat2, localized to the mitochondria and peroxisomes [108], and Yat1 and Yat2, localized to the outer mitochondrial membrane and cytosol, respectively [92, 106, 109]. Through this transport system, acetyl-CoA formed in the peroxisomes and cytosol can be fully oxidized in the mitochondrial TCA cycle. However, the shuttle system requires that carnitine is supplemented, as *S. cerevisiae* in contrast to many other eukaryotic species lacks the biosynthetic enzymes required for carnitine biosynthesis [106, 110]. The transcription of genes related to the carnitine shuttle are subjected to glucose repression [108, 109]. This fact, and the observation that carnitine supplementation fails to support growth of strains in which the PDH bypass was inactivated even in glucose-limited conditions [111], strongly suggest that the carnitine shuttle system exclusively transfers acetyl-CoA units into the mitochondria.

During growth on minimal media using C_2 carbon sources as substrate, the carbon contained in acetyl-CoA has to find its way into the mitochondria irrespective of the carnitine shuttle system described above. This requires the action of the glyoxylate cycle, which can be described as an anabolic variation of the TCA cycle occurring in the cytosol and peroxisomes [112-119]. The main features and the differences between the two pathways are highlighted in **Figure 3.** The glyoxylate cycle bypasses two CO_2-emitting reactions occurring in the TCA cycle (catalyzed by isocitrate dehydrogenase (Idh) and α-ketoglutarate dehydrogenase (Kgd)) while it invests two molecules of acetyl-CoA per cycle, yielding one molecule of C_4 dicarboxylic acid as the end product. Similar to the mitochondrial cycle, the glyoxylate cycle is initiated by the condensation of acetyl-CoA with oxaloacetate (C_4) catalyzed by Cit2, localized to the peroxisomes [120]. Citrate (C_6) is converted via aconitate to isocitrate by aconitase (encoded by *ACO1*), which also catalyzes the corresponding reaction in mitochondria. Isocitrate is cleaved to succinate (C_4) and glyoxylate (C_2) by the glyoxylate cycle-specific enzyme isocitrate lyase (encoded by *ICL1*). Glyoxylate is further condensed with another molecule of acetyl-CoA, catalyzed by malate synthase (encoded by *MLS1* and *MLS2*). Similar to the TCA cycle, malate dehydrogenase (encoded by *MDH1* in mitochondria and *MDH2* and *MDH3* in the glyoxylate cycle) transforms malate into oxaloacetate, which completes the cycle. The formed succinate is imported into mitochondria via a succinate/fumarate symporter (encoded by *SFC1*), and subsequent conversion of cytosolic fumarate to malate catalyzed by fumarase (encoded by *FUM1* for both mitochondrial and cytosolic form), is required for growth of *S. cerevisiae* on C_2 compounds [121]. Cytosolic oxaloacetate can then be used to fuel the mitochondrial TCA cycle or gluconeogenesis via a decarboxylation and ATP-dependent formation of the glycolytic intermediate PEP catalyzed by phosphoenolpyruvate carboxykinase (encoded by *PCK1*). Finally, via activity of Pyk1/2, the MPC transporters and the PDH complex, PEP is converted into mitochondrial acetyl-CoA, closing the route of cytosolic to mitochondrial acetyl-CoA supply in the absence of carnitine.

In accordance with its essential role during growth on C_2 carbon sources, activity of the glyoxylate cycle is suppressed by glucose, and activated when glucose is removed [112, 122, 123]. The nature of glucose-mediated regulation is complex, and the repressive effect is transmitted by interlinked regulatory interactions and signaling pathways, briefly reviewed by Kayikci and Nielsen [124]. The mechanism of transcriptional repression of gluconeogenesis

genes is dually regulated by the kinase Snf1. At high glucose concentrations, Snf1 is inactive and remains in the cytosol. At glucose limited conditions, Snf1 is activated by phosphorylation at which it transfers to the nucleus where it phosphorylates the transcriptional repressor Mig1. The phosphorylation releases Mig1 from the promoter of genes involved in utilization of alternative carbon sources, including the transcriptional activator Cat8 [125]. Cat8 is in turn activated by Snf1-mediated phosphorylation, and is required for transcriptional activation of majority of the genes encoding enzymes required for C_2 utilization, such as *ADH2*, *ALD6*, *ACS1*, *ICL1*, *MLS1*, *MDH2* and *SCF1* [126-128].

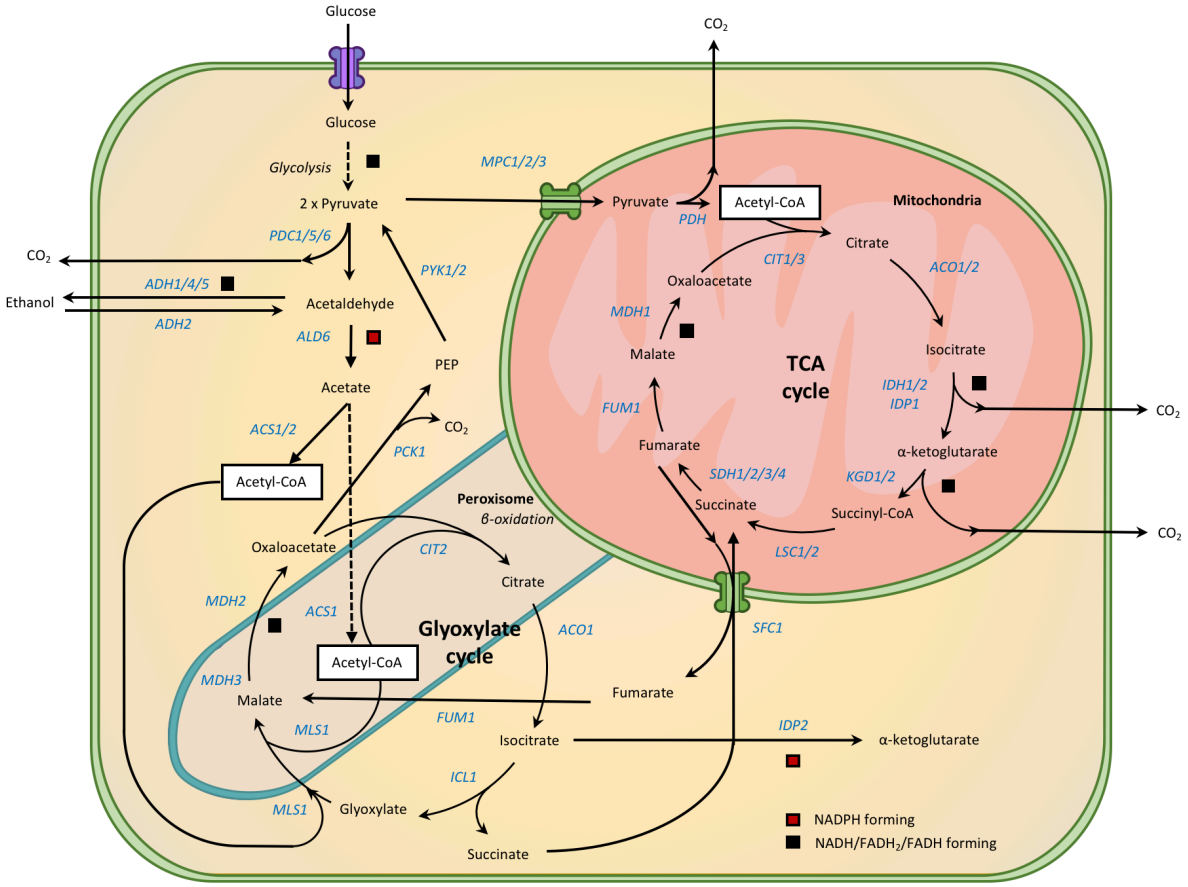


Figure 3. Major differences of the glyoxylate cycle and TCA cycle. The TCA cycle is located to the mitochondria and results in the complete oxidation of acetyl-CoA and formation of two molecules of CO_2. The enzymes of the glyoxylate cycle are located in the cytosol and peroxisomes, and result in the formation of one molecule of succinate (C_4) derived from 2 molecules of acetyl-CoA.

Acetyl-CoA and protein acetylation

Acetyl-CoA furthermore has an important role in the post-translational modification (PTM) of proteins. It acts as the donor of in protein acetylation, occurring on the ε-amino group of lysine-residues. The modification is reversible, and the addition and removal are catalyzed by lysine acetyltransferases (KATs) and deacetylases (KDACs, also commonly referred to as histone deacetylases, HDACs), respectively [94]. The by far most studied protein acetylation events are those of histones – the nuclear proteins that package DNA into structural nucleosome units. Addition of an acetyl-group to a lysine residue neutralizes its positive charge, thereby relaxing interaction of histones with the negatively charged DNA while other proteins such as RNA-polymerase gains access [129]. In this way, histone acetylation-status is a vital regulator of gene transcription.

Along with advances in the area of proteomics, it is today widely recognized that protein acetylation also has an important regulatory role over non-histone enzymes. The number of unique acetylation sites detected in human (~2500) compares with that observed for the well-known regulatory PTM phosphorylation (~2200) [130]. Also yeast proteomic studies show the broad scope of protein acetylation. Henriksen et al detected more than 4000 unique acetylation sites in *S. cerevisiae* [49], and 3160 were detected in the oleaginous yeast *Yarrowia lipolytica* [131]. Interestingly, acetylation has in various studied organisms frequently been found to occur on central metabolic enzymes [48], in which modifications rapidly could influence catalytic activity. For example, the acetylation patterns of metabolic enzymes in the prokaryote *Salmonella enterica* was shown to coordinate carbon source utilization and metabolic flux [132]. Furthermore, there is a high degree of conservation of the acetylation sites of metabolic enzymes from prokaryotes to eukaryotes [49, 133]. This implies that protein acetylation could have an important, preserved role also in metabolic regulation.

One enzyme that has strong connection to acetyl-CoA metabolism and has been shown to be deactivated by acetylation is acetyl-CoA synthetase (Acs). This feed-back regulation has been confirmed for *S. enterica* as well as in mammalian AMP-forming Acs, which if acetylated require activation by NAD^+-dependent deacetylases – known as sirtuins – for proper function [134-137]. The lysine residues that are subject to regulation in the *S. enterica* Acs are conserved in *S. cerevisiae* Acs1 and Acs2, corresponding to Lys675 and Lys637, respectively [79, 80]. Sirtuins have been suggested to have an important role in the metabolism of short chain fatty acids in yeast. This is based on the observation that a strain lacking both sirtuin genes *HST3* and *HST4* exhibits a severe growth impairment when grown on acetate or propionate, but not on glucose or a mixture of glycerol and ethanol [138]. No direct evidence of regulatory acetylation of Acs1 and Acs2 in yeast have so far been presented, however. Furthermore, there have been few attempts to evaluate the role of the individual acetylation sites detected in acetylome studies. To determine which of all detected acetylation sites that mediate a regulatory function constitutes a great challenge.

The concentration of acetyl-CoA has been shown to correlate with the acetylation status of proteins in the nucleus as well as mitochondria [50, 94, 139]. Acs2 is indicated to be the main source of acetyl-CoA for histone acetylation, and the acetyl-CoA concentration is at its highest in exponential phase, reflected in a higher degree of histone acetylation in this phase [94, 139]. In a yeast strain where *PDA1* was deleted (rendering the mitochondrial PDH-complex nonfunctional) the acetylation of mitochondrial proteins was significantly reduced while the total cellular acetyl-CoA levels decreased by 30% [50]. Methods for quantification of acetyl-CoA can currently not distinguish between the mitochondrial and the nucleocytosolic fractions. However, as mitochondria occupy as little as 1-2% of the cellular volume during exponential phase growth on glucose [140], a reasonable assumption is that the mitochondrial acetyl-CoA concentration under normal conditions is about 20-30 times higher than that of the cytosol. This agrees with the observation that mitochondrial proteins in particular are found to have multiple acetylation sites and have a higher basal level of acetylation compared to cytoplasmic proteins [49, 50].

Lysine residues are progressively acetylated non-enzymatically *in vitro* in the presence of increasing concentrations of acetyl-CoA [50]. This suggests that protein acetylation may depend on non-enzymatic processes also *in vivo*. This is especially likely in the mitochondria where no KAT have yet been identified, acetyl-CoA is present in high concentration, and the relatively alkaline pH of 8.0 is permissive for such reactions to occur [141-143]. Recent studies have shown that the two homologs SIRT3 and Hst4 in human and yeast mitochondria, respectively, are likely to have a role in suppressing acetylation levels of mitochondrial proteins which otherwise would accumulate fast and interfere with protein activity [144, 145].

2.3 Fatty acid biosynthesis and regulation

Biosynthesis of fatty acids

Acetyl-CoA acts as an important building block for cellular constituents, such as sterols and fatty acids (FAs), which both constitute essential components of the cell membrane. The overall reaction principles of FA biosynthesis (FAB) are conserved from bacteria to eukaryotes, while the enzymatic machinery differs on a structural level. In brief, the synthesis is based on a cyclic process, in which thioester-intermediates are extended by two carbons at a time through steps of 1) condensation, 2) reduction, 3) dehydration and 4) reduction. An overview of the synthesis process is depicted in **Figure 4.**

The cyclic FAB process requires acetyl-CoA and malonyl-CoA as substrates. Malonyl-CoA is directly derived from acetyl-CoA in a carboxylation reaction catalyzed by acetyl-CoA carboxylase (encoded by *ACC1*), which is dependent on ATP and biotin. The reaction is considered to be rate limiting in the synthesis of FAs.

In yeast and other eukaryotic organisms, the fatty acid synthase (FAS) is built from one or two distinct polypeptides – in *S. cerevisiae* encoded by *FAS1* (β) and *FAS2 (α)*. These polypeptide(s) comprise all enzymatic domains required for *de novo* FAB, a system referred to as type I FAS [146]. In the bacterial FAB machinery, each enzymatic domain is instead represented by an individual protein, a system referred to as type II FAS [147]. In *S. cerevisiae*, the Fas1/Fas2-complex producing the main pool of FAs is located to the cytosol, but yeast also encodes a mitochondrial system of type II essential for respiratory growth due to the mitochondrial requirement for lipoic acid [148]. The operative FAS is a large complex consisting of $\alpha_6\beta_6$ dodecamers, with six catalytic chambers in each of which eight enzymatic reactions or transfers are catalyzed [38], as described below.

In order for FAS to function, it must be activated via addition of a phosphopantetheine prosthetic group (the "pantetheine arm") of CoA to the acyl-carrier protein (ACP, located on Fas2), catalyzed by the phosphopantetheine transferase (PPT, located on Fas2). ACP has a central role throughout the synthesis process, shuttling substrate intermediates to all the catalytic centers of the FAS chamber. After activation, acetyl transferase (AT, located on Fas1) loads the acetate primer from acetyl-CoA onto the SH-terminus of the ACP pantetheine arm. The elongation cycle is initiated when the acetate primer (or the growing acyl-intermediate) is transferred to Cys1305 of the ketoacyl synthase (KS, located on Fas2). The empty ACP subsequently moves to the malonyl/palmitoyl transacylase domain (MPT, located on Fas1), acquiring a malonate from malonyl-CoA. The ACP shifts back to the KS domain which catalyzes a condensation reaction between the acetate and malonate units, releasing the CO_2 which initially was captured during malonyl-CoA formation by Acc1. Thereafter, ACP shuttles the 3-ketoacyl intermediate sequentially to the ketoacyl reductase domain (KR, located on Fas2), the dehydratase domain (DH, located on Fas1), and the enoyl reductase domain (ER, located on Fas1), producing a saturated hydrocarbon chain elongated by two carbons at the end of each cycle. The two reductase reactions require NADPH as electron donor, resulting in the consumption of two molecules of NADPH per elongation cycle.

When the length of the FA typically reaches 16 or 18 carbons, elongation termination occurs when ACP directs the acyl-chain to the MPT domain, where it is transferred to the SH-group of CoA and the FA is released as an acyl-CoA thioester – the form required for cellular metabolic utilization of FAs [38]. Acyl-CoAs are not readily soluble in the cytosol, and have been found to be non-covalently and reversibly bound with high affinity by the acyl-CoA binding protein Acb1 [149]. The protein is evolutionary well-conserved, and is believed to transport and deliver acyl-CoAs to utilizing systems in all eukaryotic cells [150, 151].

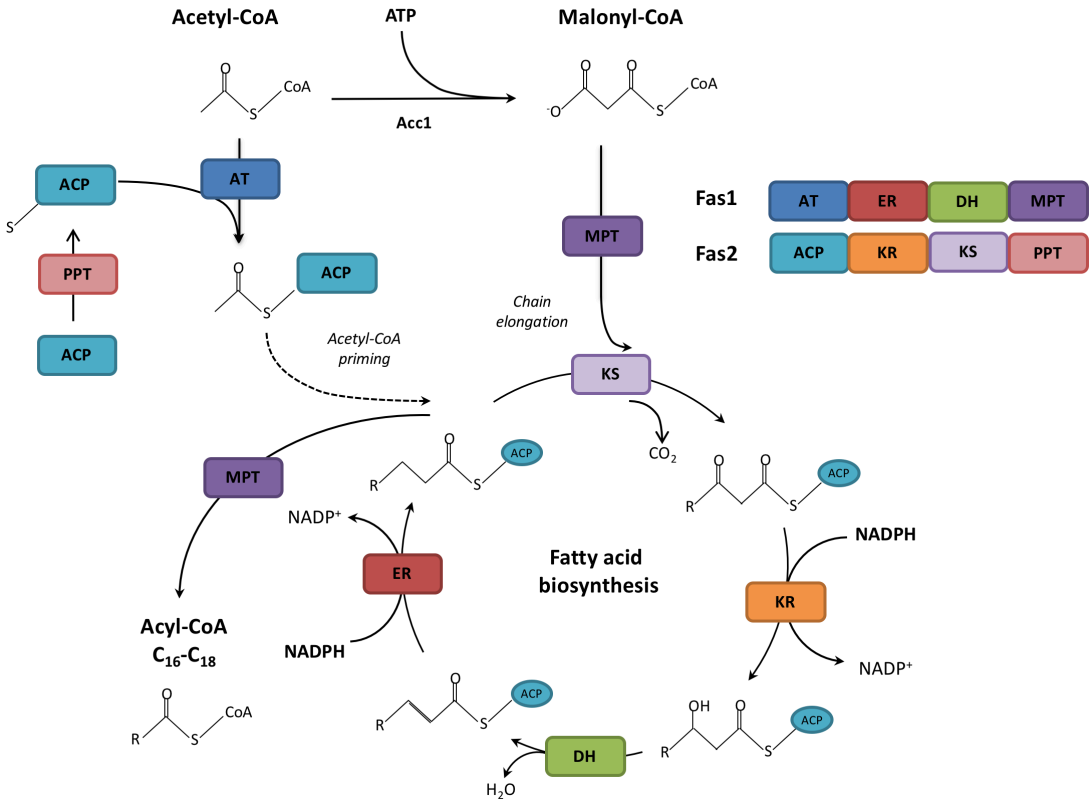


Figure 4. Fatty acid biosynthesis in *S. cerevisiae.* Adapted from Bergman et al. [152].

The fate of fatty acids

The acyl-chains have multiple destinations within cells. Of fundamental importance is the formation of membrane lipids, where the FAs are used to produce amphiphilic phospholipids with the ability to form bilayers. Around 75-80% of the FAs are desaturated by the Δ9 desaturase Ole1, residing in the endoplasmic reticulum (ER). This feature is essential to maintain membrane fluidity [153, 154]. The FA elongation system, also located to the ER, is responsible for elongating the FAS-produced $C_{16\text{-}18}$ FA species up to a length of C_{26} [155]. FAs can be stored for utilization at a later stage, in the form of sterol esters (SE) and triacyl glycerol (TAG) which accumulate in lipid droplets [156].

Once the cell requires FAs, either for membrane synthesis or as energy source, the storage lipid or phospholipid bound FAs can be released through the action of lipases [157, 158]. Both lipase-produced and exogenously fed free FAs are required to be activated to acyl-CoA species by acyl-CoA synthases (encoded by *FAA1-FAA4* and *FAT1*) prior to metabolic utilization [159]. Thus, strains devoid of acyl-CoA synthases cannot utilize FAs as a sole carbon source, and during regular growth conditions such cells, and even the double mutant *faa1*Δ*faa4*Δ, excrete free fatty acids (FFAs) into the media [160]. Acyl-CoA species can be degraded via β-oxidation, a peroxisomal processes which essentially consists of the reversed reactions compared to FAB: cyclic steps of 1) oxidation, 2) hydration, 3) oxidation and 4) thiolytic cleavage. Acetyl-CoA is the end-product of β-oxidation, and therefore be considered to be a third major route for acetyl-CoA formation in yeast (shown in **Figure 2**).

As FAs are required for membrane synthesis, production of FAs occurs throughout all growth phases. However, the proportion of lipid species are dynamically changing with growth phases, in particular after entering the diauxic shift and moving into ethanol phase [161]. Storage lipids (TAG/SE) are depleted during an initial lag-phase, and throughout exponential

phase the level of storage lipids remains low. Storage lipid formation increases when the cells enter the diauxic shift, and are again mobilized when cells enter the post-diauxic shift. The increased proportion of storage lipids observed during the diauxic shift is related to the transient quiescent phase observed at glucose depletion, where the synthesis of membrane lipids is not required [161].

Regulation of fatty acid biosynthesis

Synthesis of one molecule of octadecanoic acid (C_{18}) consumes 9 molecules of acetyl-CoA, 8 molecules of ATP (during malonyl-CoA formation), and 16 molecules of NADPH. Due to this high energy and cellular resource requirement, FAB is a process bound to be highly regulated. Transcriptional activation and repression, product inhibition and post-translational mechanisms have all been shown to influence FAs synthesis [39, 42, 162, 163].

Transcription of *ACC1* and *FAS1*/*FAS2* is controlled in coordination with many genes involved in phospholipid synthesis via the inositol-responsive upstream activating sequence (UAS_{INO})-mediated system. The system was initially characterized for regulation of the gene *INO1*, encoding inositol-3-phosphate synthase responsible for production of the lipid precursor inositol. The UAS_{INO} regulatory element is a 10 bp long sequence that serves as the binding site for the transcriptional activator complex Ino2/Ino4 [164, 165], and has been identified in the promoters of *ACC1* and *FAS1*/*FAS2* [39, 166, 167]. The transcriptional repressor Opi1 interacts with Ino2, thus inhibiting transcription of genes preceded by UAS_{INO}-containing promoters [168]. This repression is dependent on the cellular level of the phospholipid precursor phosphatidic acid (PA). At high concentrations of PA, Opi1 localizes to the endoplasmic reticulum (ER), where the PA stabilizes Opi1 interaction with the ER-membrane protein Scs2 [169].

In addition to the Ino2/Ino4 mediated transcriptional activation, *FAS1* and *FAS2* are positively co-regulated by the general regulatory factors Rap1, Abf1 and Reb1 [170]. The Fas1 protein product also directly influences expression of *FAS2*, possibly to counteract an unbalanced ratio of Fas1 to Fas2 protein [171].

Acyl-CoA species are the direct product of FAB and have been found to impact the activity of Acc1 as well as the FAS complex. The activity of Acc1 was shown to be significantly reduced in the presence of exogenously added FAs, while the repression was not observed in a strain devoid of acyl-CoA synthase activity. This led to the conclusion that the repression is mediated by the acyl-CoA species [172]. It is not completely clear how the enzymatic repression is mediated in yeast. Nevertheless, *in vitro* studies of a purified acetyl-CoA carboxylase homolog from rat showed that palmitoyl-CoA efficiently inhibits enzyme activity via an allosteric mechanism preventing the formation of an active enzyme complex [173]. Furthermore, this inhibitory effect was distinctly reduced in the presence of an acyl-CoA binding protein [174]. With regard to regulation of FAS activity, palmitoyl-CoA and stearoyl-CoA are effective inhibitors of the enzyme [162]. The inhibition is considered to be an effect of a limited dissociation of the acyl-CoA species from the enzyme following to synthesis [175]. Such a situation is likely to occur when Acb1 becomes saturated. On the contrary, acyl-CoA pools are observed to be enlarged in a strain overexpressing Acb1, thus stimulating FA synthesis [149]. The Acb1-acyl-CoA complex also has a role in regulating gene expression, as a strain devoid in Acb1 or expressing an Acb1 mutant unable to bind acyl-CoA esters results in an increased expression of *ACC1*, *FAS1* and *FAS2* among other genes involved in phospholipid synthesis [176].

Acc1 is also regulated by phosphorylation by the kinase Snf1, resulting in enzyme inhibition [41, 163]. Acc1 represents the rate-limiting step in the formation of FA synthesis, and is the same time a critical regulator of nucleocytosolic acetyl-CoA levels and therefore acetylation

dynamics [177]. Phosphorylation events catalyzed by Snf1 are dependent on the interaction of Snf1 with several factors, such as one of three regulatory subunits (Sip1, Sip2 or Gal83), and the stimulatory factor Snf4 [178]. Sip2 has been found to be acetylated by the acetyl transferase NuA4, a modification that strengthens its interaction with Snf1, which in turn inactivates Snf1 [179, 180]. The level of Sip2 acetylation correlates with the nucleocytosolic acetyl-CoA concentration and is lowered in a *snf1*Δ mutant, rendering Acc1 non-inhibited and acetyl-CoA levels low [177, 181]. In this way, it is believed that acetylation of Sip2, activity of Snf1 and Acc1, and nucleocytosolic acetyl-CoA levels form a homeostatic mechanism which would maintain acetyl-CoA concentration within acceptable limits [80].

Acc1 and Fas1/Fas2 have also been demonstrated to be acetylated at multiple residues [49]. Due to the central role of acetyl-CoA in FA synthesis as well as protein acetylation, it is tempting to assume that acetylation could have a direct regulatory role of FAB-related enzymes, analogous to that observed for acetyl-CoA synthetase in cells of bacterial and human origin. Indeed, in human cells, acetylation of the FAS has been associated with an increased level of protein ubiquitinylation and, as a result, its subsequent degradation [51]. However, no study prior to this has been conducted to determine a regulatory role of acetylations detected on the FAB-related enzymes in *S. cerevisiae*.

2.4 Cellular redox balances and cofactor biosynthesis

Cellular degradation of sugars, energy generation and biosynthesis of cellular constituents are all processes that are dependent on "redox chemistry". In a redox-dependent chemical reaction, an electron is transferred from one molecule to another, thereby changing the oxidation state of an atom in each molecule. A molecular entity that passively functions as an electron donor or acceptor in enzymatic catalysis, and otherwise remains unaffected, is referred to as a redox cofactor or coenzyme. Redox cofactors permit the cell to separate the occasion of donation and final utilization of electrons. An example is when electrons are donated to NADH during glycolysis while NADH ultimately transfers electrons to the respiratory chain or to acetaldehyde in respiratory and fermentative conditions, respectively.

Redox couples and balances

The two chief redox couples in central carbon metabolism are the nicotinamide adenine dinucleotide (phosphate) pairs NAD^{+}/NADH and $NADP^{+}$/NADPH. However, many other important redox couples exist, for example FAD/$FADH_2$ and GSSG/2GSH, the former important in cellular processes such as the TCA cycle and the latter in oxidative stress response, respectively [182]. NAD(H) and NADP(H) have distinct tasks within biological systems, where NAD(H) predominantly acts in catabolic reactions, while NADP(H) is involved in biosynthetic reactions [66]. As the cofactors merely shift between a reduced and an oxidized state, an important feature to consider is the ratio of the two partners at a given time point. The ratio is a measurement of the availability of cofactors within the system, and it regulates the ability to perform redox-dependent reactions within a cell.

As neither NAD(P)H nor $NAD(P)^{+}$ directly can traverse the organelle membranes, the redox balances are distinct in mitochondria and the cytosol. Furthermore, NAD^{+} and in particular NADH are often found in protein-bound form, which is considered kinetically and thermodynamically irrelevant [183]. Conventional analytical methods cannot distinguish between the cytosolic and mitochondrial redox pools, nor between free and protein-bound cofactors. In order to determine the free cytosolic NAD^{+}/NADH ratio, Canelas et al. designed a redox sensor based on a bacterial mannitol-1-phosphate-5-dehydrogenase expressed in the yeast cytosol, allowing to estimate the ratio through measurement of the metabolites mannitol-

1-phosphate and fructose-6-phosphate [184]. Through this method (and assuming the cytosolic pH to be between 6.5-7.0), the NAD^+/NADH ratio was estimated to be between 100-300 in aerobic, glucose limited chemostats – more than 10-fold higher than what was measured with a spectrophotometric whole-cell method. The high cytosolic ratio of NAD^+/NADH permits oxidative reactions to take place, including the glycolytic reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a $\Delta G°=6{,}3$ kJ mol^{-1} [185]. On the contrary, the NADPH/$NADP^+$ ratio is required to remain high to be able to drive anabolic reactions. Analogous to the method developed by Canelas et al., Zhang et al. designed a metabolite sensor to monitor the cytosolic NADPH/$NADP^+$ ratio in yeast, based on a bacterial shikimate dehydrogenase with high specific activity [186]. By measuring dehydroshikimate and shikimate, the *S. cerevisiae* NADPH/$NADP^+$ ratio was estimated to be around 22 and 16 in batch and chemostat culture, respectively.

Nicotinamide adenine dinucleotide synthesis

A summary of important synthesis routes and cytosolic/mitochondrial transport mechanisms of $NAD(P)^+$/NAD(P)H couples in yeast is depicted in **Figure 5**. NAD^+ can be synthesized *de novo* from tryptophan or regenerated by NAD-degradation products in a "salvage pathway". In the *de novo* pathway, nicotinic acid mononucleotide (NAMN) is formed from tryptophan in 6 enzymatic steps (catalyzed by enzymes encoded by *BNA1-BNA2* and *BNA4-BNA7*) and one non-enzymatic reaction. At this point, the *de novo* and the salvage pathways converge, and through the action of two additional enzymes (encoded by *NMA1*/*NMA2* and *QNS1*), NAD^+ is formed from NAMN [187, 188].

$NADP^+$ (and NADPH) synthesis in turn depends on phosphorylation of NAD^+ (and NADH). There are two NAD(H) kinases residing in the cytosol of *S. cerevisiae*, which primarily phosphorylate NAD^+ into $NADP^+$, encoded by *UTR1* and *YEF1,* where Utr1 appears to have the most important physiological role [189-191]. Furthermore, mitochondria also contain a NADH kinase, encoded by *POS5,* which has preference for NADH as substrate [192]. Pos5 has a vital role in mitochondrial protection toward oxidative stress, and a *pos5*Δ mutant shows respiratory defects, while a *pos5*Δ *utr1*Δ genotype results in a lethal phenotype [191].

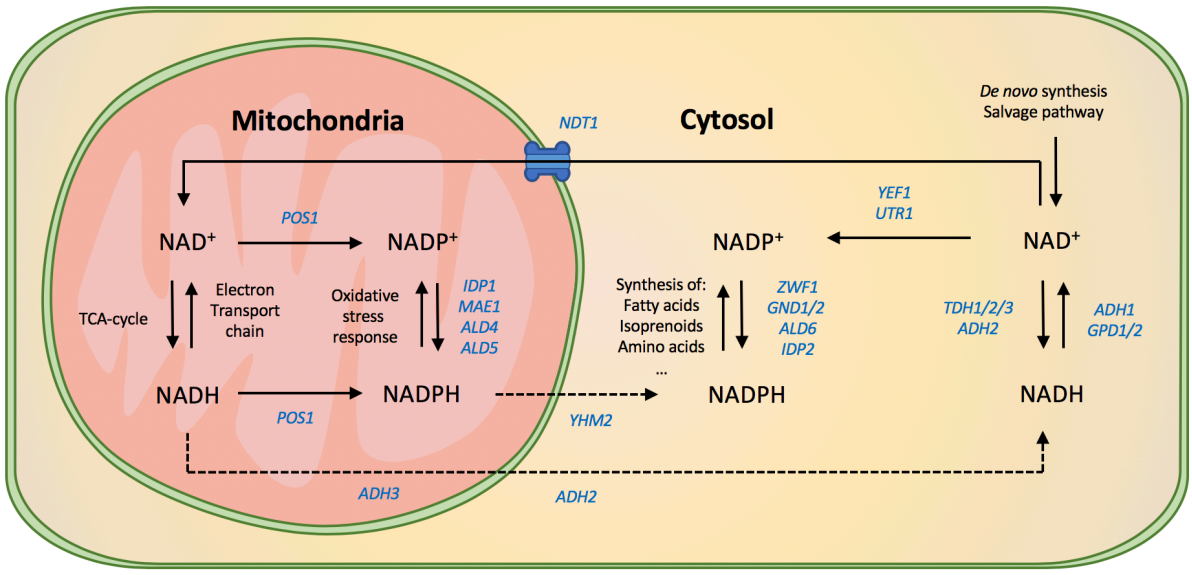


Figure 5. An overview of biosynthetic reactions influencing the redox status of *S. cerevisiae*. The dashed bars indicate genes involved in redox shuttles of the connected NAD(P)H species.

NADH-forming reactions

One of the most important NADH forming reactions in the cytosol is that catalyzed by GAPDH, resulting in 2 NADH per glucose, while activities of the TCA cycle produce 4 NADH per pyruvate entering the cycle in the mitochondria. The NADH produced by glycolysis in the cytosol is in anaerobic conditions and in glucose excess re-oxidized through the formation of ethanol. Glycerol formation, on the other hand, allow the cell to establish redox balance with excess NADH generated from the assimilation of sugar to biomass [66]. When glucose is consumed in a completely respiratory manner, e.g. in a glucose-limited chemostat, the NADH produced in the cytosol must be transferred to the electron transport chain. *S. cerevisiae* possesses three mitochondrial NADH:ubiquinone oxidoreductases which oxidize NADH and transfer electrons to ubiquinone. *NDI1* encodes a version whose catalytic site faces the mitochondrial matrix, oxidizing NADH produced via the TCA cycle. In contrast, *NDE1* and *NDE2* encode proteins with catalytic activity facing the cytoplasmic side of the mitochondrial outer membrane, and thus can act as a direct transfer of cytosolic NADH to the respiratory chain [193]. Furthermore, a redox shuttle based on ethanol-acetaldehyde diffusion over the mitochondrial membrane and mitochondrial alcohol dehydrogenase (encoded by *ADH3*) has been shown to be essential for growth in a *ndi1*Δ mutant [194]. Additionally, the mitochondrial NAD^+ carrier protein (encoded by *NDT1*) allows for transport of cytosolic NAD^+ into the mitochondria [195].

NADPH forming reactions and regulation

Cytosolic NADPH generation in *S. cerevisiae* as well as in other eukaryotes is dependent on the oxidative PPP. Two molecules of NADPH are formed per G6P being converted into R5P and CO_2, resulting from the enzymatic activity of the dehydrogenases Zwf1 and Gnd1/Gnd2. The importance of the oxidative PPP on NADPH supply is supported by the observations of *zwf1*Δ mutant strains, which become hypersensitive toward oxidative stress [196-198] and – at least in some strain backgrounds, such as S288C – methionine auxotrophic [199]. NADPH is the essential reducing component of the glutathione antioxidant protection system [200] and three molecules of NADPH (four in total, if considering the requirements of thioredoxin reductase) are required in order to convert organic sulfate to hydrogen sulfide [201, 202], which is required for methionine biosynthesis. It is therefore widely considered that the hypersensitivity to oxidative stress and methionine auxotrophy of the *zwf1*Δ phenotype both are the result of a reduced availability of NADPH. The observation that methionine prototrophy is restored in response to an availability of NADPH from an alternative source, i.e. by overexpressing the $NADP^+$-dependent acetaldehyde dehydrogenase encoded by *ALD6*, supports the latter hypothesis [203]. Ald6, as well as the isocitrate dehydrogenase encoded by *IDP2*, constitute additional cytosolic routes for NADPH generation. Ald6 is essential for growth on glucose in a *zwf1*Δ strain [203], while Idp2 has a more pronounced impact on NADPH generation during growth on non-fermentable carbon sources, when the glucose-mediated transcriptional repression of the gene is relieved [204].

In the mitochondria, there are several $NADP^+$-utilizing dehydrogenases: *ALD4* and *ALD5* encode mitochondrial acetaldehyde dehydrogenases, *MAE1* encodes malic enzyme, which catalyzes an oxidative decarboxylation of malate to pyruvate, and *IDP1* encodes a mitochondrial isocitrate dehydrogenase. Among these, the acetaldehyde dehydrogenases, in particular Ald4, have a critical role in providing NADPH in mitochondria in the absence of Pos1 [205]. As previously mentioned, the mitochondrial pool of NADPH is distinct from that in the cytosol. However, the mitochondrial transporter protein Yhm2, which exports citrate and imports α-ketoglutarate, is suggested to function as a NADPH-redox shuttle in yeast. The

export of citrate to the cytosol may result in the generation of cytosolic NADPH, providing that the citrate is converted to α-ketoglutarate (see **Figure 3**). Thus, the Yhm2 transport reaction yields a net export of reducing equivalents from mitochondria to the cytosol, supported by an observed decrease in cytosolic NADPH/$NADP^+$ ratio in a *yhm2*Δ mutant [206].

One of the most important flux diversions which can be made to influence the NADPH supply is the branchpoint of glycolysis and the oxidative PPP. *ZWF1* is considered to be constitutively expressed on various carbon sources [196, 207]. However, the flux through the node is not static, and is believed to be of a flexible type [208]. For example, roughly 16.2% of all the glucose consumed in an aerobic batch culture are shuttled via the oxidative PPP, while the corresponding number is 44.2% in an aerobic, glucose-limited chemostat, closely corresponding with the increased requirement of NADPH for biomass synthesis [209]. Zwf1 activity in crude cell free extracts is inhibited by NADPH, indicating that the NADPH concentration or the NADPH/$NADP^+$ ratio dictates the flux entering the oxidative PPP [210]. Furthermore, overexpression of *YNO1* in *S. cerevisiae* – encoding an NADPH oxidase – caused a 10-fold increase in superoxide levels, while no such increase was present in a *zwf1Δ* mutant. This further supported the idea that the flux through the oxidative PPP dynamically increases in response to the NADPH demand [211].

However, the discussed flux diversion is not solely dependent on the activity of Zwf1. This is exemplified by the processes set in motion by oxidative stressors, leading to a dynamic redirection of glycolytic flux towards the oxidative PPP. This metabolic reconfiguration is initiated via the inhibition of GAPDH in response to oxidative perturbation, occurring within seconds of exposure and thus effectively blocking the glycolytic flux and increasing the intracellular concentration of G6P [212]. Exposure to oxidative stress also activates gene expression of multiple genes within the oxidative and non-oxidative PPP, as well as other genes encoding NADPH-producing enzymes, mediated by the transcription factor Stb5 [47, 213]. However, such regulation is bound to be slower than a metabolic reconfiguration due to the requirement of mRNA and protein synthesis.

2.5 Engineering of yeast metabolism

The cell factory

The production of ethanol and penicillin by *S. cerevisiae* and the filamentous fungus *Penicillium chrysogenum*, respectively, are well-established industrial processes. They both represent examples of how microorganisms can be utilized as cell factories, catalyzing the conversion of simple carbohydrate substrates into specific metabolic products of commercial interest. Industrial penicillin-producing strains are today estimated to produce more than 100.000-fold more than the first confirmed penicillin-producing isolate [214]. Both of the above-mentioned processes exploit the natural substrate-to-product spectrum of the production organism, and the discussed optimization of the penicillin production process were primarily made via untargeted mutagenesis and optimization of cultivation parameters.

The rapid progress made in the field of genomics and genetic engineering over the recent decades now allows for a transfer of traits between organisms in a precise fashion. This has vastly expanded the industrial microorganism production portfolio, as attractive metabolic pathways of organisms which for example are problematic to culture or genetically modify can be inserted into a more suitable production host. As a result, research fields such as synthetic biology and metabolic engineering are now blooming, and researchers aim to design, create and study customized organisms with a precise tasks [215]. For example, pathway architecture can be modified to achieve a better yield of an established production process, or a newly unraveled metabolic pathway can be installed to enable commercial production of a high-value chemical.

One of the greatest challenges societies of today face is to find substitutes for the conventional oil and natural gas refineries, which provide petroleum for the transportation sector and platform chemicals and secondary chemicals for the polymer and solvent industries. Not only are these resources physically limited, but they release massive amounts of carbon dioxide to our atmosphere when extracted, processed and used. The use of microbial catalysts to produce carbon-based chemicals and fuels, e.g. of an oleochemical character, on the other hand constitutes a sustainable alternative, as carbon dioxide sequestering plants and organisms can act as the feedstock (or even as cell factories themselves) [216]. Furthermore, as discussed in **Chapter 1**, fermentation-based technologies for production of compounds based on FAs offer a much broader range of substrates to be utilized compared to the conventional chemical conversion of plant- and/or animal-based oils. Microbial communities have conquered virtually every environmental niche imaginable and have consequently adapted to metabolize numerous food sources - traits which can be transferred between organisms. Thus, current waste products from agriculture and forestry industries could be used as substrate for the production of various products if microbes can be constructed to catalyze an efficient conversion, for example yeasts engineered to utilize cellulose and hemi-cellulose as substrates [217].

Some parameters of importance to consider in the design of a cell factory are yield, titer and productivity. The yield represents how much product is obtained for a certain amount of substrate (e.g. g product/g substrate), and the titer is how much product is obtained per culture volume (e.g. g/L). The productivity in turn reflect how efficient the production process is in terms of time (e.g. g/L/h). The three parameters will influence an industrial production to a smaller or greater extent. For example, if a bulk chemical of lower value is produced, the product-on-substrate yield is required to be high, as the cost of the substrate commonly accounts for a large proportion of the total production cost. However, even if a process has a high yield it might not be optimal from a productivity point of view. For example, if the microbial conversion process takes a full week to reach completion, the cost of the production facility might outcompete the benefit of a high yield. Finally, final titer is usually a parameter of importance in the purification process, where the cost can reach high levels if the concentration in the medium is low [218].

Metabolic engineering principles

As mentioned, metabolic engineering allows us to optimize fermentation processes by introducing targeted genetic changes into the microbial catalyst. A schematic overview of the metabolic engineering process is shown in **Figure 6**. It consists of repeated cycles of design of modifications to introduce, strain construction, cultivation and sampling, and data analysis. The workflow tends to be iterative, as the analysis of the effect of an introduced genetic change often will indicate that additional genetic perturbations are required to improve the performance of the cell factory.

The “Design” step of the cycle commonly makes use of a “rational strategy” based on currently available knowledge of the chosen organism, such as metabolic reaction networks and known protein properties. The complexity of biological systems however makes this process imperfect, as decisions commonly are based on a highly simplified view of metabolism. It is becoming more common that the design step is aided by genome scale models of microorganisms. These models aim to include a large proportion of the reactions taking place within a cell and in the simplest form handle three levels of information: metabolites, reactions and genes, from which predictions of metabolic fluxes can be made. The first genome-scale model of yeast metabolism was published in 2003, which contained 1145 reactions, 825 metabolites and 708 genes [219]. From there onwards newer versions have regularly been presented with more details (e.g. including more genes/metabolites) and with different types of

constraints laid on top of the original network to achieve a better prediction of cellular behavior. An example of constraints employed are based on transcriptome data, in which the information on gene expression are used to adjust the flux passing through certain reactions. Analogously, proteomic, thermodynamic, enzyme kinetic and metabolomic constraints can be implemented to achieve better predictions [220].

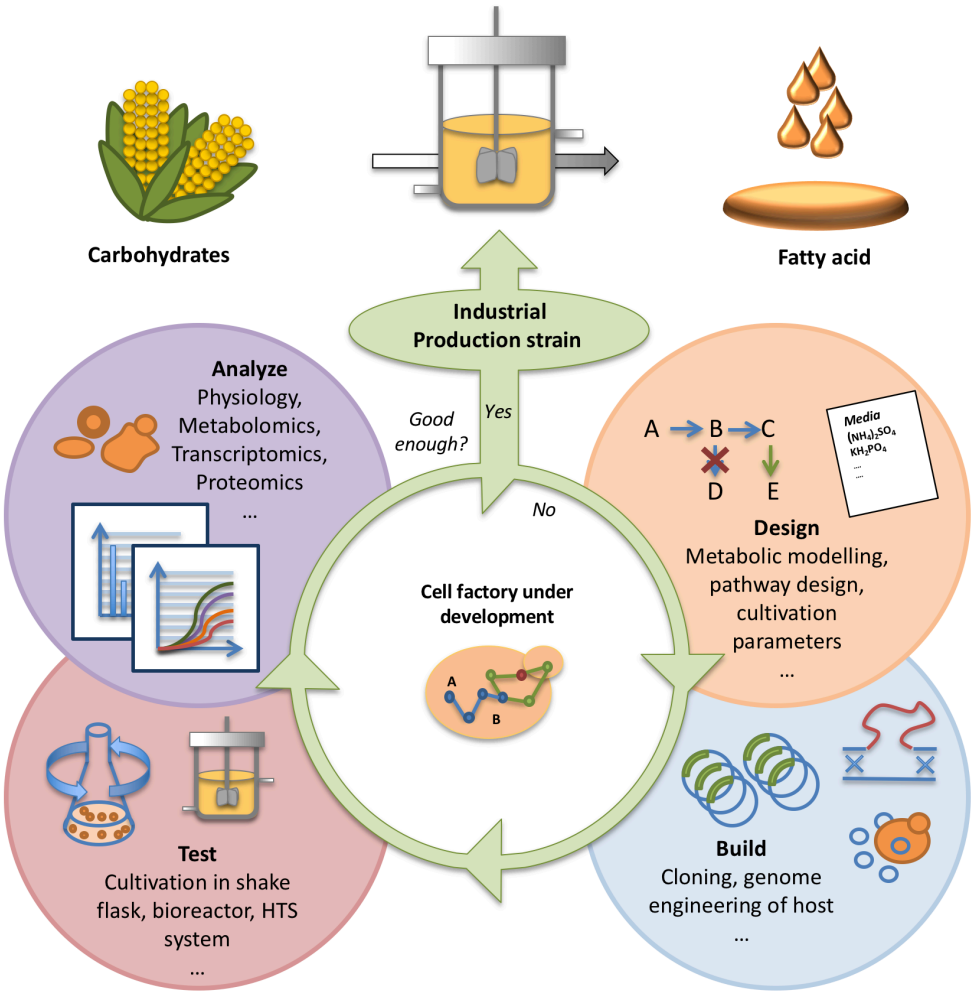


Figure 6. Overview of the biorefinery concept and the metabolic engineering cycle. Adapted from Bergman et al. [152].

The "Build" step of the metabolic engineering cycle entails to introduce the genetic change(s) into the host, either by expressing the desired construct from a plasmid or by integrating them into the host genome – a step also referred to as strain construction. In 1973, Cohen, Boyer and co-workers reported the first event of successfully engineering the bacterium *Escherichia coli*, thus developing the field of recombinant DNA technology. Shortly thereafter, techniques became available to modify the yeast *S. cerevisiae* [221, 222]. As opposed to non-homologous end joining, yeast preferentially repairs double strand breaks via homologous recombination (HR) [223]. As a result, by simply introducing a linear DNA fragments bearing homologous sequences to a specific chromosomal location, the fragment will be perceived as a double strand break and specifically incorporated into the genome. Conventionally, a type of selective marker has been required to be simultaneously incorporated in order to efficiently identify mutated cells [224]. With the advent of CRISPR/Cas9 technology – first reported for

use in *S. cerevisiae* six years ago [225] – multiple marker-less modifications can now be introduced in a single transformation [226].

Once the desired strains are constructed, the "Test" phase can be initiated. Simply put, it entails to cultivate strains in one way or another and take samples which convey information about strain performance. First tests are commonly performed in a small-scale setting. There are high-throughput systems that allow to cultivate a large number of individual strains (such as 48 or 96-well plates) at once and automatically monitor cell density and/or fluorescence. Such methods are superior in terms of a first screening of growth but limited for applications where larger sampling volumes are required. At a somewhat larger scale, falcon tubes or shake flasks are commonly utilized. However, certain cultivation parameters are hard to control in small scale cultures. For example, full oxygenation is difficult to achieve by simply swirling the culture, especially at higher cell densities. Furthermore, even if buffered medium is utilized, the pH is altered throughout the culture period.

A highly controlled experiment can be performed in bioreactors, where the parameters temperature, pH, and dissolved oxygen can be precisely monitored and adjusted along the cultivation. Furthermore, the system can be connected to a sensor analyzing the gas content of the exhaust gas, giving important information about the rate of oxygen consumption and carbon dioxide formation. The medium composition will still change over time in a batch culture, and by introducing genetic changes into strains, the growth rate will also likely be altered from the reference strain. By employing a chemostat setup, also the medium composition and growth rate can be controlled within the bioreactor.

Metabolic engineering tools and strategies

A central concept in metabolic engineering is to re-direct carbon fluxes within the cell to optimize product formation. An increased flux through a pathway of interest can be achieved through several different means, but it commonly comes down to increasing the overall enzyme activity in a particular pathway. The pathway in turn can either be "endogenous", meaning that it originates from within the concerned host organism, or "heterologous", meaning that it stems from another source that can provide the host with a novel or improved function.

The modulation of enzyme activity can be achieved by targeting different levels of regulation. One of the most common ways is to enhance the transcriptional level of the target genes, generating more messenger RNA (mRNA) which in turn gets translated into proteins. This can be accomplished by increasing the total gene copy number in the cell or utilizing strong promoters, i.e. the gene regulatory elements present upstream of the gene sequence that recruit components of the transcriptional machinery. Frequently, modulation of translational efficiency is also utilized as means to increase enzyme activity. In case of expression of heterologous genes, codon-optimization can be utilized, which optimizes the nucleotide content of the gene sequence to match the most prevalent codon usage in the receiving organism [227]. Addition of a short "Kozak consensus sequence" just upstream of the start codon can also be utilized, which enhances initiation of translation of the mRNA sequence [228]. The last, and most direct, step to influence enzyme activity is to make targeted modifications of the protein sequence to influence catalytic activity of the enzyme, for example by enhancing binding affinity of the enzyme to its substrate or by abolishing post-translational regulation. Another way is to physically bring the enzyme in closer proximity to its substrate, i.e. to enhance the local concentration of substrate – by substrate channeling-techniques.

Due to the inherent nature of metabolic networks, the product of one reaction is commonly the substrate for one or more reactions. Therefore, another common strategy to increase flux through a desired pathway is to down-regulate, or completely abolish, flux through competing pathways. A down-regulation can be achieved by replacing the native promoter of a gene with

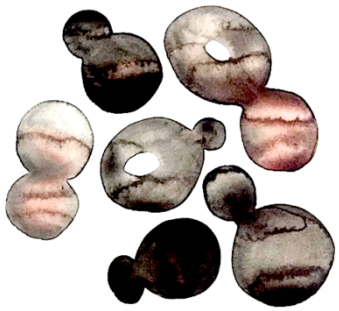
a weaker one, or introducing a mutation into a gene which limits the flux through the produced enzyme. Furthermore, if the enzyme activity is not essential for cell survival, the activity can be completely abolished, either by introducing a premature stop-codon, or by deleting the genetic material from the cell completely.

A metabolic engineering strategy commonly aims to influence the metabolism of the host at many different points simultaneously. **Table 1** presents some examples of utilized strategies when constructing cell factories. For a more comprehensive overview, the reader is referred to reviews on the topic, for example by Lee et al. [229].

Table 1. Examples of metabolic engineering strategies utilized in yeast.

Strategy	Examples of modifications to be introduced	Reference
Optimize/expand substrate utilization	Modify cells to more efficiently take up certain substrate, to co-utilize substrates, or introducing genes which enable utilization of substrates not naturally consumed by the host.	[230]
Reduce/eliminate byproduct formation	Deletion or downregulation of genes responsible for diverting flux from the targeted pathways	[31]
Increase precursor metabolite pool	Enhancing expression of genes catalyzing reactions upstream of the desired target compound to steer the carbon flux toward precursor formation (and, consequently, product formation)	[231]
Rerouting of metabolic pathways	Introduction of a heterologous enzyme which in a more beneficial way, e.g. with increased energy or carbon efficiency, can produce the sought-after product.	[232]
Co-factor engineering	Increase production of a required co-factor; alter co-factor specificity of enzymes; introducing a redox-balance reaction to maintain cellular homeostasis.	[233]
Expanding product spectrum	Introducing heterologous pathways to produce non-native chemicals; engineering endogenous enzymes to form new products.	[22]
Enhancing strain tolerance	Development of tolerance (for example in the presence of fermentation inhibitors or toxic products). Required modification is commonly identified by performing adaptive laboratory evolution/genome sequencing, followed transferring the suspected causal mutation into desired host.	[234]

3 Prospects and limitations of a phosphoketolase-based route for acetyl-CoA



3.1 Engineering acetyl-CoA metabolism

As described in the previous chapter, acetyl-CoA is a central metabolite highly relevant for a multitude of cellular processes. Acetyl-CoA is the fuel of the TCA- and glyoxylate cycle, the acetyl-donor in protein acetylation, and the immediate precursor for isoprenoid and fatty acid synthesis. The role of acetyl-CoA as the precursor for the biosynthesis of various commercially interesting products, such as fatty acids (e.g. used as/in dietary supplements, consumer products, lubricants, biofuels), isoprenoids (e.g. used as/in flavor and fragrances, dietary supplements, vitamins, pharmaceuticals, and biofuels), polyketides (pharmaceuticals), polyhydroxyalkanoates (biodegradable polymers), etc., has led to a great interest for engineering acetyl-CoA metabolism in *S. cerevisia*e as well as in other organisms. In this section a summary of various strategies explored by the metabolic engineering community to influence acetyl-CoA metabolism in *S. cerevisia*e are presented.

The most apparent way to increase acetyl-CoA supply is to increase the flux through the natural pathway towards cytosolic acetyl-CoA, namely through the PDH bypass (Pdc/Ald/Acs). An early effort elevate acetyl-CoA levels in yeast through engineering the PDH bypass were performed to positively influence the production of the isoprenoid amorphadiene [235]. The strategy was based on overexpression of the native target gene *ALD6* and expression of a mutant acetyl-CoA synthetase from the bacterium *S. enterica.* By removing an inhibitory acetylation site in the Acs (ACS_{SE}^{L641P}), a 4-fold increase in amorphadiene production was achieved. A successful extension of this strategy to promote α-santalene synthesis additionally utilized overexpression of *ADH2*, to promote ethanol conversion to acetaldehyde, and a deletion of *CIT2* or *MLS1*, to prevent the acetyl-CoA of flowing into the glyoxylate cycle [236].

However, the strategies adopted in the above-mentioned studies do not resolve the fact that the native route to form cytosolic acetyl-CoA from pyruvate in *S. cerevisiae* results in loss of CO_2 and energy. The carbon loss results from the decarboxylation of pyruvate catalyzed by Pdc1/5/6, and energy expenditure (ATP→AMP) occur during activation of acetate by Acs1/2. As both carbon and energy loss limit the theoretical yield of the biorefinery process, multiple studies have investigated acetyl-CoA generating routes with potential to increase the theoretical yield of product formation in *S. cerevisiae*, commonly of heterologous origin. A visual and stoichiometric overview of these strategies are shown in **Figure 7** and **Table 2**, respectively.

ATP-citrate lyase (ACL)

ATP-citrate lyase (**ACL**) is responsible for production of cytosolic acetyl-CoA from citrate in many higher eukaryotic species, and is a hallmark feature of many oleaginous yeasts [237]. Since the citrate originates from mitochondrially produced acetyl-CoA, the pathway is not able to circumvent carbon loss. In addition the reaction requires ATP, but forms ADP instead of AMP, resulting in a lower net consumption of energy compared to the native yeast pathway. The pathway has been utilized in several different metabolic engineering strategies in *S. cerevisiae* to positively influence production of fatty acids, n-butanol and mevalonate [238-241]. An ACL from *Mus musculus* was either expressed in a wild type or in strains in which mitochondrial forms of isocitrate dehydrogenase were disrupted to increase the citrate concentration, and both strategies were found to have moderate positive effects on fatty acid production [238]. Lian et al. enhanced titers of n-butanol by two-fold when they expressed the ACL from the oleaginous yeast *Y. lipolytica* [239]. More recently, several ACLs from different organisms were expressed and compared in *S. cerevisiae*. The ACL from the filamentous fungi *Aspergillus nidulans* were shown to have a 4-6 fold higher activity than previously evaluated enzymes, including those from *M. musuculs* and *Y. lipolytica*. In combination with other modifications, the expression of the *A. nidulans* ACL significantly increased mevalonate production in S. [240], and has subsequently fruitfully been used to enhance FA synthesis [15].

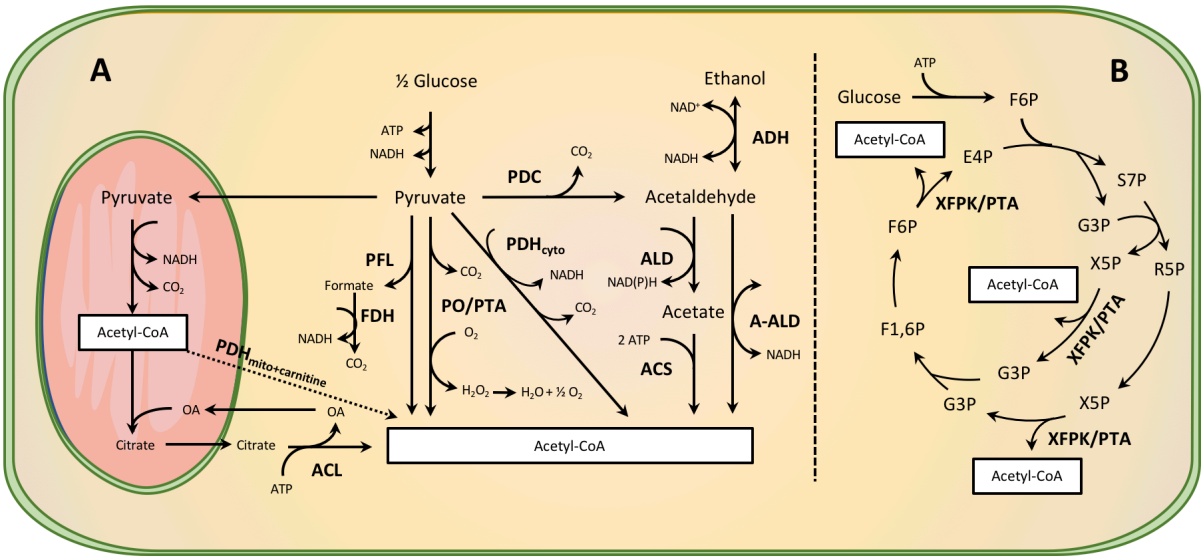


Figure 7. Strategies to produce cytosolic acetyl-CoA in *S. cerevisiae* via **A)** the native (PDC/ALD/ACS) or the alternative routes PFL/FDH, PO/PTA, PDH_{cyto}, A-ALD, $PDH_{mito+carnitine}$ and ACL and **B)** a suggestion of XFPK/PTA route in combination with endogenous reactions catalyzed by the gluconeogenetic enzyme Fbp1 and enzymes from the PPP and glycolysis. Adapted from van Rossum et al. [43].

Cytosolic expression of pyruvate dehydrogenase complex (PDH_{cyto})

An alternative way to improve energy efficacy in cytosolic acetyl-CoA synthesis is to express a pyruvate dehydrogenase complex in the cytosol (**PDH_{cyto}**). This solution enables an ATP-independent synthesis of cytosolic acetyl-CoA from pyruvate. The strategy was published by two independent research groups in 2014. The approach of one group involved either co-targeted the mitochondrial *S. cerevisiae* PDH complex to the cytosol or heterologous expression of PDH from *E. coli* [239], while the second group utilized a PDH from *Enterococcus faecalis* [242]. Lian et al. claimed that both PDH-expression strategies led to increased acetyl-CoA levels, had a positive effect on the production of n-butanol, and supported growth of a PDC-

negative strain [242]. The researchers however did not comment on the expression of genes involved in lipoylation of the PDH-subunit E2, nor did they supplement their cultures with lipoic acid. Kozak et al., on the other hand, found that expression of the PDH-subunits from *E. faecalis* exclusively supported growth in an ACS-negative strain providing genes encoding putative lipoate ligases (able to activate subunit E2) were expressed and lipoic acid was added to the medium. During aerobic and anaerobic batch culture, the growth rate of the modified strain was 0.35 h^{-1} and 0.30 h^{-1}, respectively, while the wild type strain had a growth rate of 0.42 h^{-1} and 0.33 h^{-1} [239].

Carnitine-dependent export of mitochondrially produced acetyl-CoA ($PDH_{mito+carnitine}$)

As described, the PDH complex offers an ATP-neutral route to form acetyl-CoA from pyruvate, which in minimal media does not contribute to the cytosolic pool due to the impermeability of the mitochondrial membrane to acetyl-CoA. Even if carnitine is present and the acetyl-carnitine transport is active, the transport machinery appears to be unidirectional, favoring acetyl-CoA import to the mitochondria [106, 110, 111]. A recent study successfully evolved yeast for carnitine-dependent export of mitochondrial acetyl-CoA to the cytosol [243]. Laboratory evolution was performed in an ACS-negative, carnitine supplemented yeast strain that 1) constitutively expressed the yeast CATs and 2) contained PDH_{cyto} that could be inactivated at omission of lipoic acid. This allowed to create a provisional condition where the strain was dependent on mitochondrial synthesis for its cytosolic acetyl-CoA requirements, and thus for growth. Adaptive evolution of this strain yielded mutants able to grow on glucose in the absence of lipoic acid, but not in the absence of carnitine. Another research group recently reconstructed a heterologous route for carnitine biosynthesis from *Neurospora crassa* in *S. cerevisiae*, and successfully demonstrated synthesis of carnitine when the precursor trimethyl-lysine was supplemented [244]. Thus, further development of these ideas could potentially provide a platform for a more energy efficient provision of cytosolic acetyl-CoA supply in yeast.

Table 2. Overall stoichiometry of acetyl-CoA formation from glucose

Pathway	Stoichiometric function
Native	½ Glucose + $2NAD(P)^+$ + ATP + CoA + H_2O → acetyl-CoA + $2(NAD(P)H + H^+)$ + CO_2 + ADP + P_i
ACL	½ Glucose + $2NAD^+$ + CoA → acetyl-CoA + $2(NADH + H^+)$ + CO_2
$PDH_{cyto\ /\ mito+carnitine}$ **A-ALD** **PFL/FDH**	½ Glucose + $2NAD^+$ + ADP + P_i + CoA → acetyl-CoA + $2(NADH + H^+)$ + CO_2 + ATP + H_2O
PO/PTA	½ Glucose + NAD^+ + ADP + P_i + CoA + ½ O_2 → acetyl-CoA + NADH + H^+ + CO_2 + ATP + $2H_2O$
XFPK/PTA	⅓ Glucose + ⅓ ATP + CoA → acetyl-CoA + ⅓ (ADP + P_i) + ⅔ H_2O

Acetylating acetaldehyde dehydrogenase (A-ALD)

Acetylating acetaldehyde dehydrogenase (A-ALD) also provides an ATP-neutral conversion of pyruvate to acetyl-CoA and is common in many prokaryotes. The enzyme catalyzes a reversible, NAD^+-dependent conversion of acetaldehyde to acetyl-CoA, and has in yeast previously been expressed to promote the opposite reaction in order to enhance ethanol yield from xylose and acetate [245, 246]. Kozak et al evaluated four heterologous, codon-optimized A-ALD candidates in an ALD-negative (*ald2*Δ*ald3*Δ*ald4*Δ*ald5*Δ*ald6*Δ) yeast and found that all candidates supported relatively fast growth [232]. However, the expression was linked to a

decreased biomass formation, believed to be caused by an accumulation of the toxic intermediate acetaldehyde in the strains. At the same time, calculations of ΔG´ suggests that an accumulation of acetaldehyde indeed is a thermodynamic requirement for the reaction to form acetyl-CoA under *in vivo* conditions.

Pyruvate formate lyase (PFL)

Pyruvate formate lyase (**PFL**) constitutes an additional ATP-independent route of acetyl-CoA formation from pyruvate, a reaction that additionally produces formate. In order to avoid negative effects mediated by formate, PFL should be co-expressed with (or expressed in a host containing activity of) formate dehydrogenase (FDH), which oxidizes formate to CO_2 using NAD^+ as electron acceptor. The catalytic activity of PFL depends on an activating enzyme (PFL-AE), that abstracts a hydrogen atom from its active site – an activity which in turn is dependent on flavodoxin as electron donor. The radical makes PFL sensitive toward oxygen and the enzyme is therefore most suited for anaerobic processes. PFL and PFL-AE were first expressed anaerobically in *S. cerevisiae* in 2009, during which formate accumulation was observed [247]. Kozak et al expressed several PFL candidates with their corresponding PFL-AE, and observed anaerobic growth on glucose of the candidates from *E. coli* and *Lactobacillus plantarum* in an ACS-negative strain, reaching 73% of that observed in the ACS-positive reference strain [232]. PFL and PFL-AE from *E. coli* have also been co-expressed with flavodoxin and/or ferredoxin proteins from *E. coli*. The study concludes that the electron donors enhance the performance of PFL-expressing strains, and the system as a whole improves the growth of a PDC-negative mutant even in aerobic conditions [248].

Pyruvate oxidase and phosphotransacetylase (PO/PTA)

The flavoprotein pyruvate oxidase (PO) can also catalyze an ATP-neutral conversion of pyruvate to acetyl-CoA if co-expressed with a phosphotransacetylase (PTA) – both commonly found in bacteria. The PO utilizes an oxygen- and inorganic phosphate-dependent oxidation of pyruvate to acetyl-phosphate (AcP), CO_2 and H_2O_2. The PTA in turn catalyzes the reversible conversion of AcP and CoA into acetyl-CoA and P_i. Detoxification of the hydrogen peroxide commonly occurs in organisms through catalase activity. The biggest difference of the PO-dependent and PDH_{cyto}/A-ALD/PFL-dependent pathways is the requirement for molecular oxygen and the generation of fewer reducing equivalents. A PO from *Aerococcus viridans* and a PTA from *S. enterica* were recently expressed in a PDC-negative strain of *S. cerevisiae* that substantially improved growth and increased the production of the acetyl-CoA dependent products farnesense and 3-hydroxypropanoate (3-HP) [249].

Phosphoketolase and phosphotransacetylase (XFPK/PTA)

The last route to have been used to promote cytosolic acetyl-CoA synthesis in *S. cerevisiae*, and which this thesis largely focused on, is based on the combined action of a phosphoketolase (XFPK) and a PTA (described in PO/PTA section). XFPK enzymes can catalyze either one or both of the redox-, carbon- and energy-neutral conversions of xylulose-5-phosphate (X5P) and fructose-6-phosphate (F6P) according to:

xylulose-5-phosphate + P_i → *glyceraldehyde-3-phosphate* + *acetyl-phosphate*
fructose-6-phosphate + P_i → *erythrose-4-phosphate* + *acetyl-phosphate*

Phosphoketolases (EC 4.1.2.9 and EC 4.1.2.22) are ThPP dependent enzymes with important metabolic roles in some species of fungi, in heterofermentative and facultative heterofermentative lactic acid bacteria (LAB), and in bifidobacterial species [250]. In particular, the F6P-specific XFPK is considered to be a taxonomic marker for the *Bifidobacteriaceae* family, due to its central role in the so-called "bifid-shunt" [251]. From the XFPK-produced AcP, PTA can catalyze the following reversible reaction:

acetyl-phosphate + CoA ←→ *acetyl-CoA + P_i*

Both the XFPK and the PTA reaction (in direction of acetyl-CoA formation) are thermodynamically favorable during standard biochemical conditions, with $\Delta G_R°' = -49.9$ to -63.2 kJ mol^{-1} for the XFPK-catalyzed reactions, and $\Delta G_R°' = -9.8$ kJ mol^{-1} for the PTA reaction [252]. As indicated in **Figure 7B**, The XFPK/PTA route can in combination with activity of enzymes of the non-oxidative PPP, glycolysis and the gluconeogenic enzyme fructose-1,6-bisphosphatase theoretically enable complete carbon conservation in the production of acetyl-CoA from fructose-6-phosphate [253]. However, glucose must initially be phosphorylated by hexokinase – a reaction which consumes ATP – to yield F6P in *S. cerevisiae*. This results in a negative ATP yield when XFPK/PTA is used to produce acetyl-CoA from glucose (**Table 2**). Therefore, some carbon must pass through glycolysis to produce ATP, preventing complete carbon conservation.

The XFPK/PTA route was initially introduced into yeast in combination with A-ALD to enhance ethanol fermentation from xylose [245]. Thereafter it has been successfully implemented to enhance production of acetyl-CoA-derived compounds such as polyhydroxybuturate (PHB) and fatty acid ethyl esters (FAEE) [32, 45]. The XFPK used in the studies by de Jong et al. and Kocharin et al. originates from *A. nidulans*, and the flux through the heterologous enzyme had previously been confirmed in *S. cerevisiae* by ^{13}C-based metabolic flux analysis [44]; however, the activity of the enzyme was not directly evaluated.

Comparative advantage of the XFPK/PTA route in acetyl-CoA and fatty acid formation

As shown in **Table 2**, the different routes for acetyl-CoA formation differ with respect to acetyl-CoA, ATP, NAD(P)H and CO_2 yield on glucose [43].

The ATP yield of the native route is negative (–1 ATP), as the ATP produced in glycolysis per ½ glucose is hydrolysed to AMP, that cellularly corresponds to a consumption of two equivalents of ATP. As discussed in the previous section, the XFPK/PTA by-passes the ATP-producing steps of glycolysis, and hence the route has a negative ATP-yield ($-^1/_3$ ATP per $^1/_3$ glucose), while all other routes have a neutral (ACL) or positive ATP-yield on glucose. The XFPK/PTA pathway is on the other hand the only route for acetyl-CoA formation circumventing production of CO_2, which also is an important factor to consider in optimizing product-on-substrate yield.

Furthermore, the XFPK/PTA route is alone in offering a redox-neutral synthesis of acetyl-CoA, a fact which influences the attainable yield of various compounds [43]. An NADH surplus obtained in glycolysis and through the various acetyl-CoA forming pathways needs to be re-oxidized to obtain redox balance. Thus, if NADH is not consumed during product formation it is required to be oxidized via other processes, such as respiration which in turn generate large amounts of ATP. In order to optimize a production process using a cell factory a slightly positive ATP yield on glucose is optimal, which can be used for cellular maintenance. Conversely, a large positive ATP yield will on the other hand divert glucose toward biomass formation, resulting in a decreased product-on-substrate yield.

An additional aspect to consider in the specific case of production of fatty acids is the requirement of NADPH. The native PDH bypass supplies 1 NADPH per acetyl-CoA formed through the action of Ald6, while the remaining pathways depend on a re-routing of carbon through the oxidative PPP for cytosolic NADPH generation.

The undesirable effects of the requirement to re-oxidize surplus NADH through respiration and completely rely on the oxidative PPP to generate NADPH become obvious when inspecting the calculation of the theoretic yield of different acetyl-CoA formation pathways for production of the fatty acid palmitic acid. While the NADH-producing pathways with neutral or positive ATP yield slightly decreases the yield compared to the native pathway (0.194 vs. 0.203 $mol_{product}/mol_{substrate}$), the XFPK/PTA pathway increases the yield to near 87% of the theoretical maximum (0.226 vs. 0.261 $mol_{product}/mol_{substrate}$) [43]. However, the highest yield is calculated to be obtained if a combined approach is employed, where the XFPK/PTA pathway should be used to produce 65% of the acetyl-CoA while the remaining 35% should stem from an ATP-independent pathway, corresponding to 89% of the maximum yield [43].

3.2 Evaluating different phosphoketolases (Paper I)

With the potential benefits the XFPK/PTA pathway could offer fatty acid synthesis, we set out to optimize and study certain aspects of the pathway. Stochiometric analysis is unquestionably a useful tool to understand the prospects as well as limitations of a certain pathway in a metabolic engineering scenario. However, such analyses assume that there is no limitation in enzyme fluxes and no interfering reactions. Reality unfortunately often tells a completely different story, where thermodynamic constraints, enzyme kinetics and competing cellular reactions greatly influence a metabolic pathways functionality.

Our initial goal was to find XFPK candidates with high catalytic activity and different substrate specificities. For example, since F6P is present at higher concentration in cells during growth on glucose [254], a higher flux through the XFPK could likely be achieved by utilizing an enzyme with high specificity toward F6P. As previously mentioned, XFPK enzymes with preference for X5P are common in heterofermentative lactic acid bacteria and enzymes with preference for F6P are common in bacteria of the *Bifidobacteriaceae* family. The recombinant pathway thus has potential to be increased considerably by investigating enzyme candidates from such sources. We decided to compare nine different enzyme candidates, including the XFPK from *A. nidulans* previously observed to positively influence production of metabolites depending on acetyl-CoA. Of the eight remaining candidates, three were from the *Bifidobacterium* genus – *B. adolecentis*, *B. breve*, *B. lactis* – and five from the Firmicute phylum: *Clostridium acetobutylicum*, *Leuconostoc mesenteroides*, *Lactobacillus paraplantarum*, and two candidates from *Lactobacillus plantarum*. The enzyme selection was in part based on that XFPK function had been verified by previous studies [245, 253, 255-260]. Furthermore, we wanted to test candidates from different bacterial families, and to include enzymes evaluated in published metabolic engineering strategies for benchmarking. The enzyme nomenclature used throughout the thesis follows the initial of the genus and the species-specific name of the origin of the enzyme, e.g. Xfpk(BB) for the phosphoketolase from *B. breve*.

Figure 8 shows a percent identity matrix of the investigated XFPK candidate amino acid sequence. As expected, the degree of similarity of the enzymes from the *Bifidobacterium* genus was high (minimum of 85% identity), while the distance was larger for most candidates falling within the Firmicute phylum (identity between 52-68%), and a comparison between the two mentioned bacterial groups returns an identity between 43-48%. Most dissimilar to all other XFPK candidates is the enzyme from *A. nidulans*, with a maximum of 37-41% homology.

Plasmids carrying the codon-optimized XFPK sequences were transformed into the *URA3*-lacking laboratory yeast strain CEN.PK 113-5D [261]. The parent plasmid pSP-GM1 [262] was co-transformed and evaluated alongside with the other strains as a control.

	Xfpk (AN)	Xfpk (BA)	Xfpk (BL)	Xfpk (BB)	Xfpk (LM)	Xfpk (CA)	Xfpk (LP2)	Xfpk (LP1)	Xfpk (LPP)
Xfpk (AN)	100	38.74	37.4	37.94	36.91	41.26	38.61	39.68	39.68
Xfpk (BA)	38.74	100	85.82	84.85	42.89	48.15	47.39	46.34	45.96
Xfpk (BL)	37.4	85.82	100	94.91	42.77	46.88	46.75	45.57	45.19
Xfpk (BB)	37.94	84.85	94.91	100	43.4	47.01	47.52	46.34	45.96
Xfpk (LM)	36.91	42.89	42.77	43.4	100	51.72	55.54	59.47	58.96
Xfpk (CA)	41.26	48.15	46.88	47.01	51.72	100	62.8	59.41	58.78
Xfpk (LP2)	38.61	47.39	46.75	47.52	55.54	62.8	100	67.94	67.3
Xfpk (LP1)	39.68	46.34	45.57	46.34	59.47	59.41	67.94	100	98.73
Xfpk (LPP)	39.68	45.96	45.19	45.96	58.96	58.78	67.3	98.73	100

91-100%
71-90%
51-70%
31-50%
0-30%

Figure 8. Percent identity matrix of investigated phosphoketolase candidates.

To find functionally expressed enzymes and to distinguish different levels of affinity of enzyme candidates to X5P and F6P, respectively, used an *in vitro* assay based on a modified version of the established ferric hydroxamate method [263]. Enzymatically produced AcP is in the reaction converted to ferric acetyl hydroxamate, appearing as a brownish coloration quantifiable at 505 nm, as indicated in **Figure 9A**. Crude cell-free extracts from strains expressing the nine XFPKs were either provided with F6P or R5P and inorganic phosphate as substrates. R5P was used as a substitute for X5P due to a market shortage, but as crude cell-free extracts and not purified enzymes were utilized, we relied on the endogenous yeast enzymes Rki1 and Rpe1 to interconvert R5P to X5P, as indicated by a previous study [253].

The calculated specific activities were normalized to the total protein content of the added crude cell-free extracts, and the combined results of the X5P and F6P assay are shown in **Figure 9B**. Seven out of the nine evaluated candidate XFPKs formed significant levels of AcP compared to the control pSP-GM1 using both X5P (assumed to be formed from R5P) and F6P as substrates. Two tested candidates did not display activity in either of the two assays, namely Xfpk(AN) and Xfpk(LPP). The highest activity was observed for Xfpk(CA) and Xfpk(LM), both showed a specific activity using X5P as substrate close to 1.0 U / mg total protein, while their corresponding specific activity using F6P as substrate was considerably lower – around 0.2 U / mg total protein. Xfpk(BB) together with Xfpk(BA) showed the highest activity on F6P – close to 0.5 and 0.4 U / mg total protein, respectively. Furthermore, these enzymes displayed a relatively high activity toward X5P, approximately 0.8 and 0.6 U / mg total protein for Xfpk(BB) and Xfpk(BA), respectively. In terms of total measured specific activity, Xfpk(CA), Xfpk(LM) and Xfpk(BB) all summed up to around 1.2 U of XFPK-activity/mg total protein. Candidate Xfpk(BL), previously expressed in yeast [245], had a sum of roughly 0.5 U / mg total protein.

As Xfpk(AN) was expressed with positive results in previous metabolic engineering strategies [32, 45], the negative results of Xfpk(AN) and Xfpk(LPP) were potentially due to an inherent insensitivity of the enzyme assay. During the optimization of the assay, we did observe a “bleaching” of the AcP standard curve when it was incubated with a mixture of protein extracts compared to when protein extract was left out (data not shown), indicating that the AcP was being degraded. The degradation effect should have been low due the addition of phosphatase inhibitor NaF to the reaction mixture. However, reaction rates of XFPK-candidates with lower activity could possibly have been matched by that of the (NaF-inhibited) endogenous phosphatases.

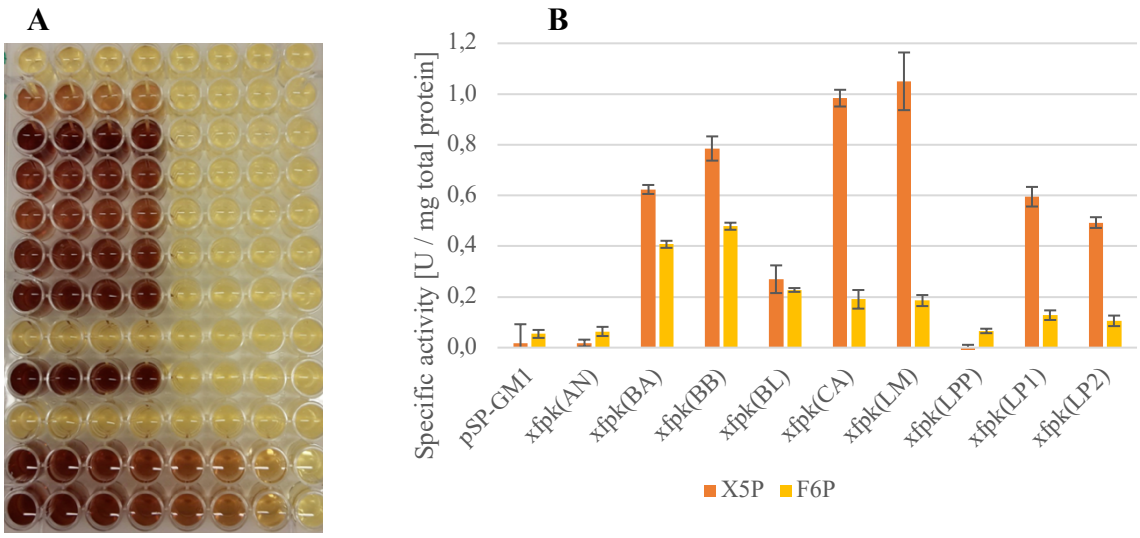


Figure 9. *In vitro* enzyme assays of XFPK activity. **A)** Physical outcome after 30 min incubation of assay reagents with crude XFPK-expressing cell-free yeast extracts, where formed AcP is converted to brownish ferric acetyl hydroxamate. **B)** Specific activities of the nine tested Xfpk enzymes with respect to substrates X5P (supplied as R5P) and F6P. Adapted from Bergman et al. [264].

Due to the observation that AcP was degraded in the presence of crude cell-free extracts, (clearly observed during a parallel development of an i*n vitro* assay to detect PTA activity, during which NaF was omitted from the reaction, see **section 3.4**), we researched recent patent literature and found that the endogenous glycerol-3-phosphatases encoded by *GPP1* and *GPP2* likely were responsible for a large proportion of the hydrolysis of AcP to acetate [265].

In order to determine the validity of this claim, we constructed *gpp1*Δ, *gpp2*Δ and *gpp1*Δ*gpp2*Δ deletion mutants and used crude cell extracts prepared thereof in a phosphatase-assay, where AcP was provided as the sole substrate. Indeed, we observed that yeast possesses an efficient enzymatic pathway to degrade AcP, and that a large proportion of this activity could be attributed to the Gpp1 and Gpp2 phosphatases (**Figure 10**). After 60 min of incubation, the AcP signal was completely lost in the wild type strain CEN.PK 113-5D. A single deletion of *GPP2* had little impact on the overall AcP degradation, retaining 12% of the original AcP concentration after 60 minutes, while the corresponding value for the *gpp1*Δ mutant was 66%. In comparison, 93% of the original AcP amount was retained after 60 min incubation with the *gpp1*Δ*gpp2*Δ sample.

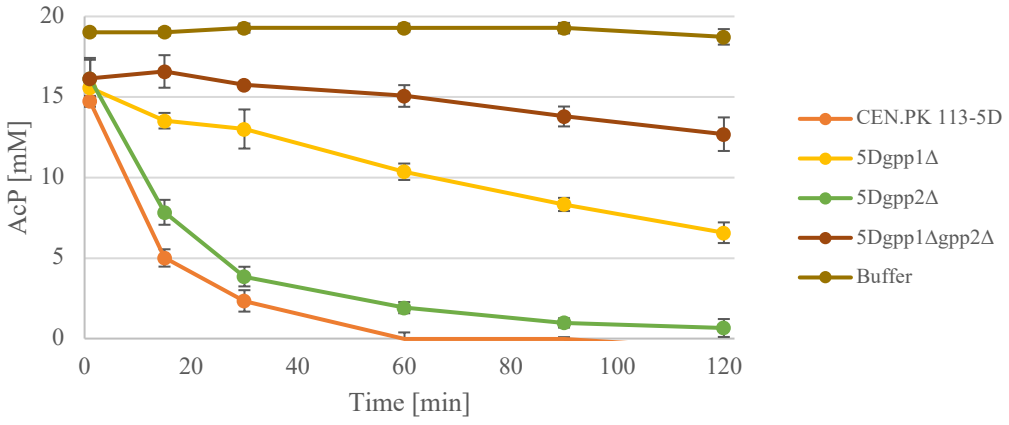


Figure 10. AcP degradation in crude cell-free extracts of CEN.PK113-5D and corresponding *gpp1*Δ, *gpp2*Δ and Δ*gpp1/*Δ*gpp2* deletion strains monitored over time. Adapted from Bergman et al. [264].

Based on an efficient natural conversion of XFPK-produced AcP to acetate, also indicated by Sonderegger et al. [245], we hypothesized we could determine which XFPK candidate had the highest *in vivo* activity by monitoring acetate levels over time. We selected the three XFPK candidates that showed relatively equal and high total activities in the *in vitro* enzyme assay – Xfp(LM), Xfpk(CA) and Xfpk(BB) – as well as Xfpk(AN) for further evaluation.

The strains carrying pSP-GM1 and XFPK-harboring plasmids were cultivated in shake flask cultures until stationary phase was reached in all strains. The maximum specific growth rate (μ(max)), maximum levels of acetate accumulation, and complete acetate and OD_{600} profiles are shown in **Figure 11A and B**. XFPK expression indeed led to an increase in acetate accumulation in all strains with expected high enzymatic activity, where Xfpk(BB) showed a 2.4-fold increase compared to the control. The fact that strains expressing Xfpk(BB) appeared to have a higher *in vivo* activity than strains expressing Xfpk(LM) and Xfpk(CA), even though they all showed a similar total activity level *in vitro*, is likely related to its higher activity on F6P. F6P is present at higher intracellular concentrations than X5P during glucose consumption [254], and the total substrate pool should thus be larger for enzyme with higher preference for F6P. No significant difference in acetate accumulation was however observed in the strain encoding Xfpk(AN), however, indicating that the use of the enzyme candidates with higher activity has potential to significantly enhance acetyl-CoA derived product formation.

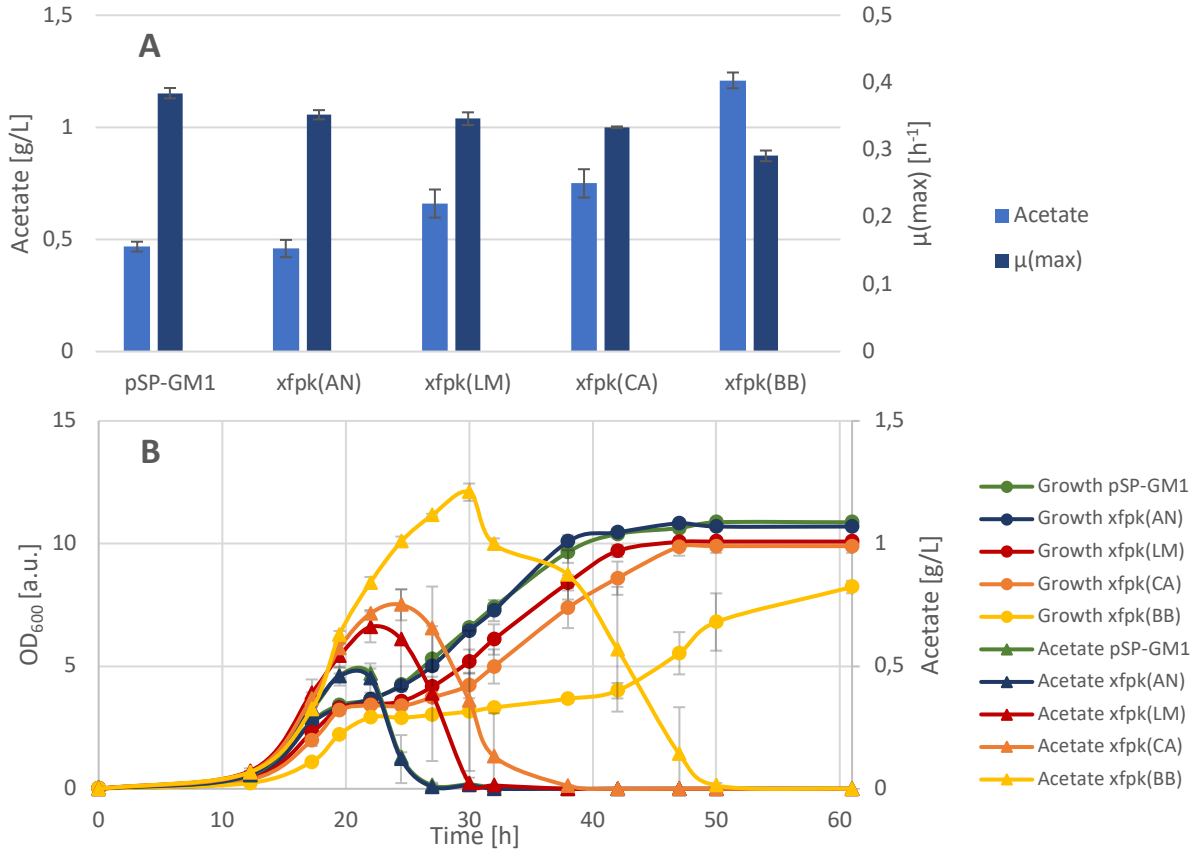


Figure 11. A) Maximal acetate accumulation and maximum specific growth rate measured in cultivations of Xfpk-expressing strains. **B)** Acetate and OD_{600} profiles during whole cultivation**.** The strains were cultivated in minimal media with 2% glucose in biological triplicates. Adapted from Bergman et al. [264].

Phosphoketolase expression of enzyme candidates with higher activity was also coupled to a decreased growth rate, reduced biomass formation and prolonged diauxic shift. Xfpk(BB) – likely to have the highest *in vivo* activity based on the acetate production profile – also displayed the most negatively influenced growth profile, with a final biomass yield and μ(max) of approximately 75% of that observed for the control.

Acetate is a weak organic acid, which dissociates into a negative anion and a proton at the near neutral pH conditions in the cell. In order to keep pH homeostasis, these ions are required to be actively pumped out of the cell via ATP-dependent transport processes. At low pH, the non-dissociated form of the acetic acid will form, that may passively diffuse back into the cell. This creates a futile cycle were ATP is consumed, referred to a proton or weak organic acid uncoupling [218]. Furthermore, in order to consume the Gpp1/Gpp2-produced acetic acid when glucose is depleted, ATP is required for its activation into acetyl-CoA. Thus, the cell is likely to encounters a situation which demands increased cellular utilization of ATP. Simultaneously, XFPK-dependent diversion of flux from glycolysis should result in a loss of ATP production, as steps of the ATP-generating portion of lower glycolysis will be bypassed.

3.3 Characterizing effects of phosphoketolase expression (Paper II)

Based on the initial observations of how XFPK expression influenced yeast growth, we decided to characterize the effects of expression in greater detail in **Paper II**. Common within the field of metabolic engineering is to introduce multiple genetic modifications simultaneously and evaluate the combined effects on cellular behavior and productivity. Using this approach, aspects of how the individual genetic changes influence the cell factory might be overlooked. In particular, expression of heterologous enzymes should be of particularly high concern, as these are likely to influence cellular physiology in unforeseen ways. For example, the XFPK product AcP is a to yeast non-native compound that potentially can be utilized by native processes or enzymes in an unconstrained manner. The ability of Gpp1 and Gpp2 to degrade the compound is one example, but potentially other effects could also influence the behavior of the cell. For example, the attenuated formation of acetate after glucose depletion (**Figure 11B**) indicates that AcP is present at elevated concentrations within the cell for a significant amount of time. In bacteria, AcP is considered a key contributor to protein acetylation and the compound has been shown to acetylate proteins non-enzymatically *in vitro* [266]. Thus, elevated levels of AcP may potentially influence yeast gene expression and metabolism [80].

The characterization was performed by cultivating the control strain and XFPK expressing strain (xfpk(BB)) in bioreactors, allowing us to control parameters such as gas flow, stirring speed, pH and temperature during batch culture, and even growth rate during chemostat culture. The strains were initially cultivated in batch mode with 2% glucose at pH 5, and after the cultures had reached the ethanol phase, glucose-limited chemostat mode was initiated with a dilution rate of $D=0.1\ h^{-1}$ and a glucose concentration in the feed of $7.5\ g\ L^{-1}$.

Based on continuous measurements of cell dry weight, OD_{600} and HPLC during exponential phase of the batch phase and steady state chemostat conditions, the physiological parameters listed in **Table 3** were calculated.

Table 3. Physiological parameters calculated for control and strain xfpk(BB) in batch and chemostat mode.

	Batch		**Chemostat**	
Strain	***Control***	***xfpk(BB)***	***Control***	***xfpk(BB)***
μ(max) (h^{-1})	0.353 ± 0.008	0.266 ± 0.006	0.104 ± 0.002	0.103 ± 0.003
Y(x/s) (gCDW/gGlucose)	0.135 ± 0.00	0.123 ± 0.00	0.479 ± 0.01	0.409 ± 0.01
q(Glucose) (mmol $gCDW^{-1}$ h^{-1})	-14.42 ± 0.36	-12.19 ± 0.39	-1.18 ± 0.06	-1.36 ± 0.03
q(EtOH) (mmol $gCDW^{-1}$ h^{-1})	21.46 ± 0.75	17.26 ± 0.52	-	-
q(Acetate) (mmol $gCDW^{-1}$ h^{-1})	0.61 ± 0.04	1.38 ± 0.03	-	-
q(Glycerol) (mmol $gCDW^{-1}$ h^{-1})	1.13 ± 0.03	0.62 ± 0.04	-	-
q(Pyruvate) (mmol $gCDW^{-1}$ h^{-1})	0.13 ± 0.01	0.15 ± 0.00	-	-
q(Succinate) (mmol $gCDW^{-1}$ h^{-1})	0.02 ± 0.0	0.01 ± 0.0	-	-
q(CO_2) (mmol $gCDW^{-1}$ h^{-1})	30.01 ± 0.32	28.16 ± 0.97	2.91 ± 0.19	3.34 ± 0.09
q(Biomass) (mmol $gCDW^{-1}$ h^{-1})	14.37 ± 0.31	10.81 ± 0.25	4.13 ± 0.31	4.07 ± 0.25
q(O_2) (mmol $gCDW^{-1}$ h^{-1})	-5.49 ± 0.53	-7.63 ± 0.76	-2.61 ± 0.20	-3.35 ± 0.12
Carbon balance	107% ± 2%	107% ± 2%	99% ± 3%	92% ± 1%

Strains were grown in biological quadruplicates in minimal media with 2% glucose during batch phase and 0.75% glucose in the feed in chemostat conditions, pH 5. Values shown correspond to averages of quadruplicate measurements ± standard deviation.

Similar to our observations from shake flask cultivations, μ(max) and biomass yield were reduced in strain xfpk(BB), approximately 20% and 10% lower compared to the control, respectively. The glucose uptake rate was reduced by 15% in strain xfpk(BB), which complicates a direct comparison of the calculated metabolic fluxes. Thus, in order to visualize how the relative carbon fluxes differed between the two strains, the q-values in **Table 3** were normalized to glucose uptake rate and entered into a schematic figure of yeast metabolism, shown in **Figure 12**. Thus, even though the flux toward CO_2 is decreased in xfpk(BB) in Table 3, the value in Figure 12 suggests a slight relative increase (38.5% versus 34.7%). If the CO_2 stemming from respiratory activity is estimated as the difference between the total amount and that produced from pyruvate decarboxylation in the cytosol (e.g. half of the normalized carbon flux toward ethanol), it indicates that the respiratory activities are increased by approximately 50%. This correlates well with an observed increase in O_2 consumption: q(O_2) was calculated to be 5.5 mmol $gCDW^{-1}$ h^{-1} for the control and 7.6 mmol $gCDW^{-1}$ h^{-1} for xfpk(BB), indicating that XFPK expression leads to a higher respiratory demand during growth on glucose.

Glycerol formation was significantly reduced in strain xfpk(BB) compared to the control, possibly due to a reduced requirement to re-oxidize a surplus of NADH as XFPK redirects flux from lower glycolysis. Expression led to an increased pyruvate excretion (50% when considering the normalized fluxes), indicating a lower flux into the mitochondria or over pyruvate decarboxylase. The most pronounced increase in flux in strain xfpk(BB) was observed for acetate, where approximately 3.8% of the total carbon was directed towards acetate formation, compared to 1.4% in the control. The higher rate of acetate formation indirectly implies that XFPK-activity is present, as AcP is degraded by Gpp1 and Gpp2.

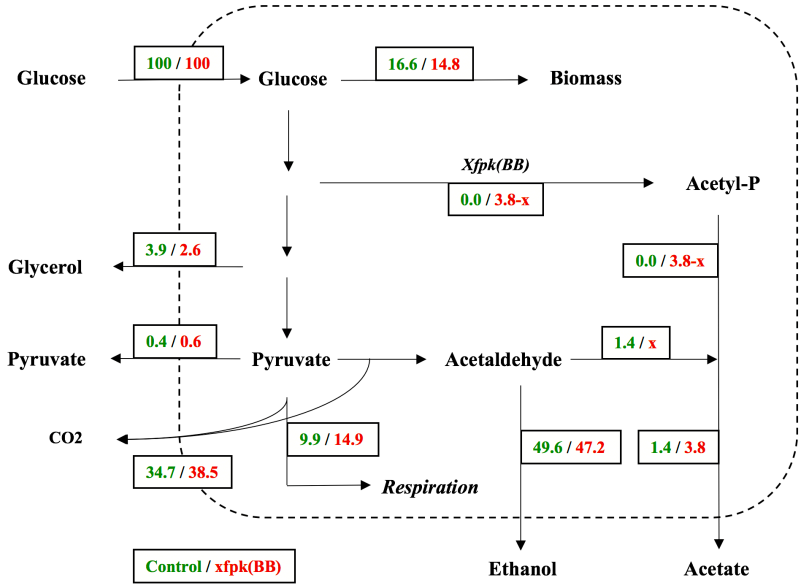


Figure 12. Carbon fluxes (in % C-moles) of exponentially growing strains normalized to glucose uptake rate. Flux towards respiration is estimated as the difference between flux toward CO_2 and half the flux toward ethanol. Fluxes corresponding to below 0.1% of the total flux are not shown in the figure. The strains were grown in biological quadruplicates with 2% glucose at pH 5. Adapted from Bergman et al. [267].

When behavior of xfpk(BB) and the control were compared in steady state conditions (**Table 3**, right panel), no accumulation of fermentative products were detected in any of the strains, supporting that the growth was fully respiratory. As indicated in **Table 3**, the biomass yield was reduced by 15% in strain xfpk(BB), matched by an increased CO_2 production rate and O_2 consumption. As acetate was not accumulated in the strains, it is unlikely that the lower biomass yield observed in strain xfpk(BB) was due to an additional ATP expenditure on cellular maintenance. Rather, the flux diversion of glucose from glycolysis – leading to a lower ATP yield on glucose – in combination with the ATP requirement to activate the Gpp1/2-formed acetate to acetyl-CoA was the cause of the reduced biomass formation.

The flux passing through the Xfpk(BB) enzyme during batch conditions can be estimated as the difference in acetate flux between xfpk(BB) and the control strain, corresponding to a moderate amount of 2.4% of the total carbon entering the cell (**Figure 12**). However, this estimate assumes that the acetate pool is left "untouched" until all glucose is depleted. Co-consumption of glucose and acetate is not considered to be typical for *S. cerevisiae*, but it has been observed previously for CEN.PK strains [268].

During chemostat conditions, the difference in acetate flux could not be used to estimate flux through Xfpk(BB), as no fermentative metabolites accumulated. The rate of O_2 consumption and CO_2 production for the control strain were in good agreement with that of a previous study cultivating CEN.PK 113-7D in glucose at the same conditions [269]. The same study also reported on the corresponding flux values when the strain was grown on an equal molar concentration of acetate as carbon source. Thus, we can hypothetically consider that the substrate source of Xfpk(BB) was a mixture of glucose and acetate, and the proportion of each substrate a linear combination of the gas flux observed under glucose and acetate conditions. This corresponds to a situation where approximately 12% of the carbon respired is provided in the form acetate, which indirectly would stem from XFPK activity. As acetate produced by Xfpk in the chemostat represents an initial loss of ATP when glucose is activated by hexokinase (compared to the situation were acetate is provided as substrate directly), the calculated value of 12% is likely an overestimation.

In order to determine the influence of proton decoupling of the produced acetate on the negative growth characteristics seen in strain xfpk(BB), we set out to investigate the growth profiles of both strains at pH 4 and 6 at which the HA-form of acetic acid should be present at 85% versus 5% (pK_a=4.76). pH-controlled batch cultivations were conducted in biological duplicates with 2% glucose. An overview of the complete cell and acetate accumulation profiles are shown in **Figure 13**. For both strains, the maximum acetate concentrations obtained at pH 6 were about two to three-fold of those seen at pH 4 (Figure 13, right graph). Under both pH conditions, maximum acetate accumulation in xfpk(BB) was more than two-fold higher than the control, the CO_2-production rate was higher for xfpk(BB) compared to the control at both pHs, while xfpk(BB) had a higher O_2 production rate compared to the control at pH 4 (see supplementary data file to **Paper II**).

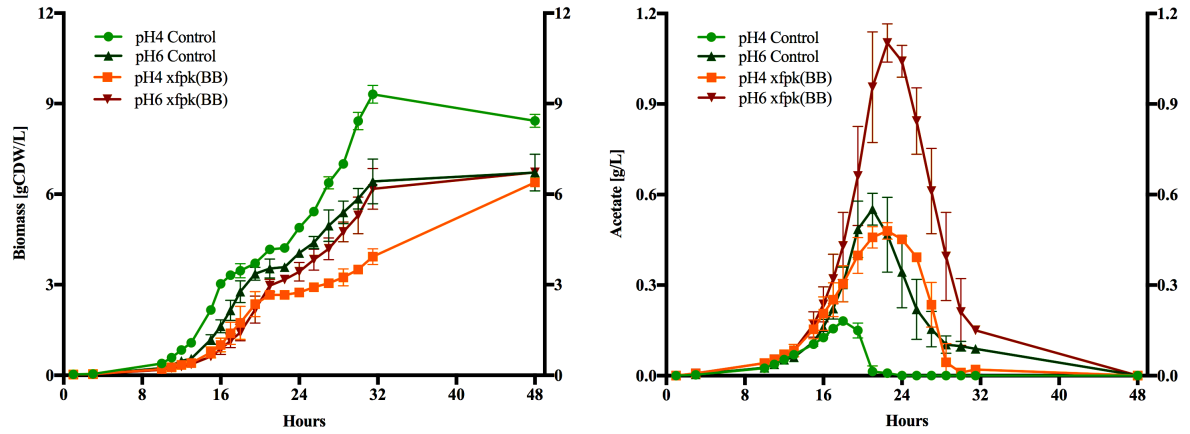


Figure 13. Biomass and acetate accumulation of control and phosphoketolase expressing strain (xfpk(BB)). Strains were cultivated in biological duplicates in pH-controlled (pH 4 and pH 6) batch cultivations in minimal medium with 2% glucose. Error bars are ± standard deviation. Adapted from Bergman et al. [267].

During exponential growth on glucose an increased pH had a minor negative influence on μ(max) in both strains, and the biomass yield for the control strain was near to unchanged. In contrast, at pH 6, the biomass yield was significantly increased for xfpk(BB) compared to its pH 4 counterpart, from 89% to about 95% of that of the control in glucose phase – indicating a more energy efficient growth. As the maximum specific growth did not increase, this suggests that the parameter is dependent on other expression-related effects, likely by the flux diversion from glycolysis. After glucose was depleted from the media, an increase in pH reduced the biomass yield of the control, resulting in a final biomass yield drop to about 80% compared to growth at pH 4. At the same time, xfpk(BB) grew faster at pH 6 than that at pH 4, reaching a growth rate almost equal to the control at pH 6, with a concurrent significant biomass yield increase (Figure 13, left graph) The positive effects of an increased pH observed for strain xfpk(BB), suggest that proton decoupling influenced the physiology of strain xfpk(BB) at lower pH, and that an increased pH reduced the ATP burden related to ionic transport.

We also investigated how Xfpk(BB) expression influenced the intracellular level of sugar phosphates during batch and chemostat conditions. Multiple sugar phosphates taking part in reactions of glycolysis and PPP – including the direct substrates and co-products forming during AcP formation – were quantified using an LC-MS approach. The absolute levels of the quantified amount of the metabolites during chemostat mode are shown in **Figure 14** expressed as μmol per g cell dry weight (μmol/gCDW). The observed trends in sugar phosphate concentrations associated with phosphoketolase expression were found to be similar during batch and chemostat cultivations. Even though the glucose uptake rate was about 10-fold lower in the chemostat versus the batch cultivation, the intracellular concentrations only decreased by about 50% (data not shown, see supplementary data file to **Paper II**)

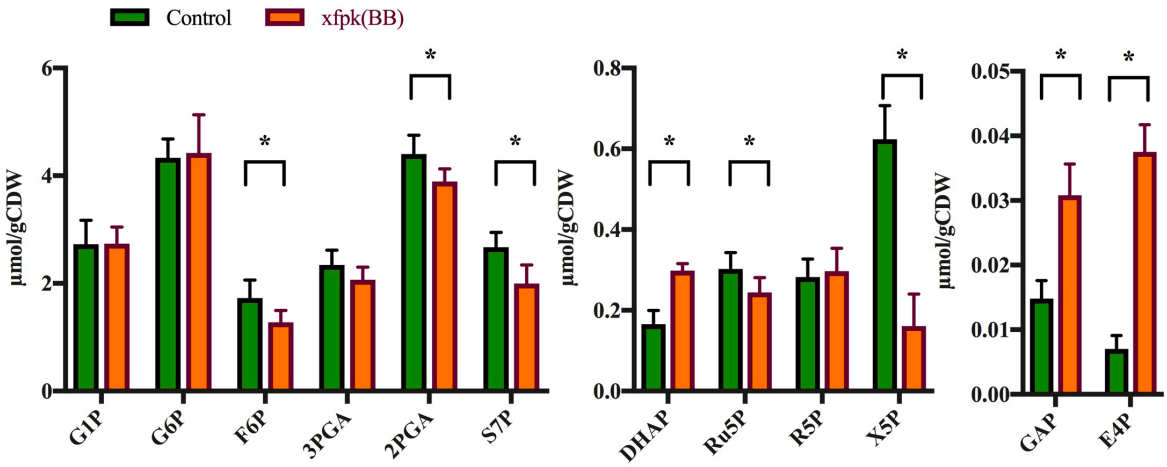


Figure 14. Quantification of sugar phosphates in the control strain and xfpk(BB) grown in glucose-limited chemostats (D=0.1). Strains were cultivated in biological quadruplicates. Significant changes are indicated with asterisks (* $p<0.05$), error bars equal ± standard deviation. Adapted from Bergman et al. [267].

Some of the most notable changes in sugar phosphate concentrations are a strong decline in the XFPK substrate X5P, a 75% reduction compared to control, while F6P was more moderately affected – corresponding to a 25% reduction compared to the control. The intracellular F6P levels were confirmed to be approximately 3-fold higher than those of X5P in the control strain, indicating that the Xfpk(BB) consumes close to equal amounts (0.45 µmol/gCDW) of both substrate pools. Considering that X5P is present in lower concentration *in vivo*, it correspond well with our observed *in vitro* activities of Xfpk(BB), suggested to be approximately 3:2 for X5P:F6P (**Paper I / Figure 9**).

The XFPK-products GAP and E4P showed a 2- and 5.4-fold increase compared to the control, respectively, giving further confirmation of *in vivo* activity and dual substrate specificity. The large gap between molar difference of consumed substrate (450 pmol/gCDW) and formed product (15-30 pmol/gCDW) can likely be explained by thermodynamic forces ensuring that the intracellular pools of GAP and E4P remain low. For example, GAP is isomerised to DHAP by triose phosphate isomerase (Tpi1) – a reaction with an equilibrium ratio of about 1:20 [185] – which is in agreement with the 2-fold increase of DHAP concentration observed in strain Xfpk(BB). Significant changes in sugar phosphate concentrations were also observed for Ru5P and S7P which are located in proximity to the substrates/products within the metabolic network (see figure **Figure 1**). The common product of the two phosphoketolase catalyzed reactions is acetyl-phosphate, which unfortunately could not be quantified with the utilized method, but its continuous formation was indirectly measured as enhanced levels of acetate in the batch phase (**Figure 12**).

As a last aspect of characterization, we also studied the global transcriptional response of Xfpk(BB) expression in yeast by conducting RNAseq analysis of the chemostat cultivated strains, and performing a differential expression analysis. 2,024 genes with an adjusted p-value smaller than 0.01 and absolute $\log_2$ fold change (|LFC|) larger than 0.1were identified. From this group, differentially expressed genes (DEGs) with a |LFC|>0.5, |LFC|>1.0 and |LFC|>1.5 corresponded to 447, 120 and 47, respectively. **Figure 15** highlight DEGs involved in the central carbon metabolism with a padj<0.01.

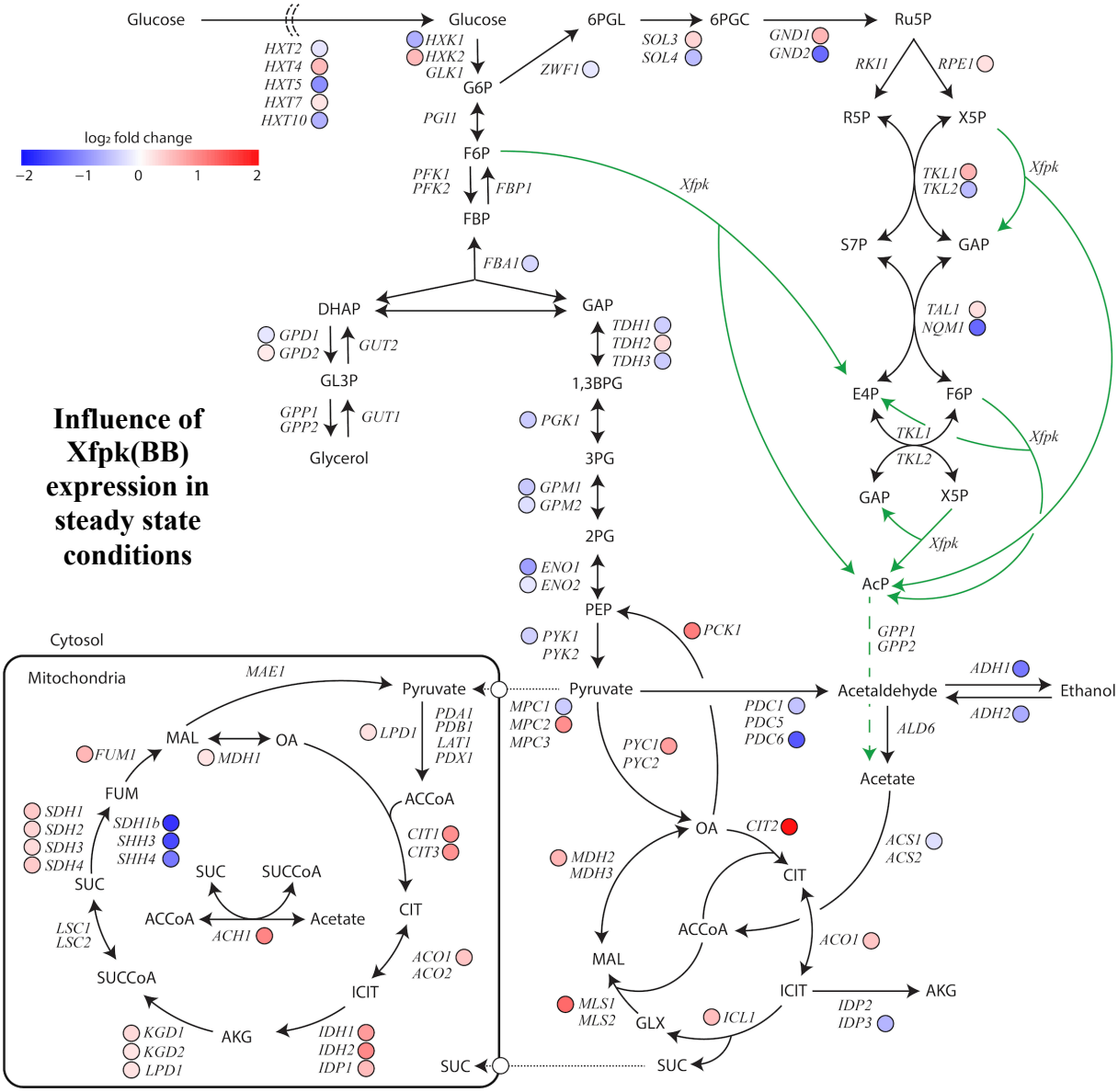


Figure 15. Transcriptional responses to phosphoketolase expression of central carbon metabolism genes expressed as $\log_2$-fold changes (padj<0.01). Extent and type (down/up) of the fold change is indicated by shading of blue (down) and red (up) color. Samples were taken from glucose-limited chemostats in biological quadruplicates. Adapted from Bergman et al. [267].

Xfpk(BB) expression had a large influence on gene expression in central carbon metabolism. Genes encoding enzymes catalyzing reactions in glycolysis were in general weakly downregulated. Pyruvate decarboxylase genes *PDC1* and *PDC6* and alcohol dehydrogenases *ADH1* and *ADH2* were downregulated, possibly an effect to reduce flux through acetate. *ACS1*, responsible for the activation of acetate to acetyl-CoA in the cytosol, was counterintuitively weakly downregulated in strain Xfpk(BB). However, as no extracellular acetate was detected in any of the strains, the activation of acetate does not appear to be limiting in the chemostat.

Multiple DEGs related to utilization of non-fermentable carbon sources – such as acetate – were found. In part, *CIT2* and *MLS1* – catalyzing the condensation of cytosolic acetyl-CoA with oxaloacetate and glyoxylate, respectively – were together with other key enzymes in the glyoxylate cycle and gluconeogenesis strongly upregulated. A vast majority of genes encoding TCA-cycle enzymes located in the mitochondria were upregulated, as well as *ACH1,* whose protein product can activate acetate directly to acetyl-CoA in the mitochondria and has in

important role in growth on acetate [97]. And even though the carnitine-shuttle system cannot be utilized during growth on minimal media [270], the genes related to acetyl-CoA/carnitine transport across intracellular membranes (*CRC1*, *YAT1*, *YAT2* and *CAT2*) were all upregulated.

A gene set analysis highlights that cellular processes such as mitochondrial translation, ATP synthesis coupled transport, cristae formation, mitochondrial respiratory chain complex IV assembly and aerobic respiration were distinctly upregulated (data not shown, see supplementary data file to **Paper II**). These changes further confirm that strain Xfpk(BB) and an increased respiratory demand, likely due to the net ATP loss from a flux diversion from glycolysis and the ATP requirement of metabolizing acetate instead of pyruvate.

Also, within the PPP, a majority of genes were identified as differentially expressed. However, paralogs encoding iso-enzymes for certain catalytic steps were regulated in different directions. If the FPKM values obtained for these paralogs are added together, the net effect is an upregulation of GND- and TKL-transcripts while the other changes are considered insignificant ($p<0.01$, two-sided T-test). As most of the observed expression changes related to the glyoxylate cycle, gluconeogenesis, TCA cycle and respiration can be traced back to a co-consumption of acetate, we wondered if the "acetate trigger" was responsible also for the changes in PPP gene expression. On the contrary, growth on acetate has correlated with a downregulation of several genes within in the PPP when compared to growth on glucose, hypothesized as a response to produce NADPH via other sources, such as Idp2 [269]. Thus, the observed net positive GND/TKL-expression change could potentially be an effect of altered intracellular levels of sugar phosphates, but studies investigating the transcriptional regulation with respect to metabolite levels are lacking to support this.

An interesting observation is that expression of multiple genes related to uracil biosynthesis were highly upregulated while the gene *URA3* was strongly downregulated in xfpk(BB). The host strain is auxotrophic for uracil, and a copy of the native *URA3* gene is expressed from the 2µ-plasmid to ensure vector-retention during cultivation in minimal media. Thus, it appears as if the cell actively keeps the plasmid present at a low-copy number in order to minimize the damage of the heterologous phosphoketolase. Indeed, when including the xfpk(BB) sequence in the genome sequence to which the RNAseq reads are aligned to, it shows that xfpk(BB) was expressed at levels lower than the 60 most highly expressed yeast genes even though expressed from the *TEF1* promoter on a multicopy plasmid, while the native *TEF1* gene was expressed among the top 20 genes. This suggest that a higher flux through the enzyme could be possible if expressed in a way that ensures that toxicity induced by acetate is minimized.

3.4 Insights of combined phosphoketolase and phosphotransacetylase expression

The characterization of XFPK expression in yeast conducted in **Paper II** highlighted the need to prevent acetate formation in order to overcome growth-related effects from weak organic acid uncoupling. In **Paper I** we showed how this can be achieved by deleting *GPP1* and/or *GPP2*. In order to complete the heterologous pathway, an efficient phosphotransacetylase (PTA) able to convert the formed AcP to acetyl-CoA is required. This subchapter presents unpublished results covering the search for an efficient PTA and insights from co-expression of XFPK and PTA.

Initially, we attempted to conduct an *in vitro* assay to find efficient PTA candidates. This work was conducted before we were aware of the function of Gpp1/Gpp2 in breaking down AcP. To establish a working method, we used a yeast strain carrying plasmid pSP-GM1 encoding a PTA from *Salmonella enterica* (Pta(SE)) and a negative control strain carrying an empty plasmid (NC). We started by employing a method developed by Klotzsch et al. in which AcP, CoA and enzyme are provided to the same reaction, followed by continuous detection of

thioester bond formation at 233 nm [271], which we however failed to implement successfully. We then tested if the ferric hydroxamate method [263] described in **Chapter 3.2** could be utilized as an alternative to detect PTA activity. All the components of the Klotzsch-reaction (buffers, AcP, CoA and protein extract) were incubated at 37°C for 30 min, after which the components of the ferric hydroxamate method converting AcP to ferric acetyl hydroxamate were applied to the samples. This resulted in an end-point read-out on how much AcP had been consumed (corresponding to a decrease in brownish coloration), results of which are shown in **Figure 16**.

The method returned the opposite results to what we expected. The results clearly demonstrated the endogenous ability of yeast to degrade AcP, as nearly all added AcP had been consumed in NC-samples. Compared to the XFPK assay performed in **Paper I**, the Klotzsch-reaction to detect PTA activity did not contain the phosphatase inhibitor NaF. These were the experiments conducted leading us to understand how efficient the native ability to degrade AcP was, and the unfavorable effect it should have on pathway efficacy.

Figure 16. A) Ferric acetyl hydroxamate method to detect AcP in samples incubated with crude-cell free extracts from strains expressing PTA from *Salmonella enterica* (Pta(SE)) or not (NC). A) **B)** Evaluation of CoA addition. Confirmation that Pta(SE) prevents AcP degradation exclusively in the presence CoA.

Even though we concluded that the ferric hydroxamate method was suitable to monitor activity of the PTA, it could nonetheless be clearly demonstrated that the enzyme interacted with AcP and prevented its breakdown when both AcP and CoA were added (**Figure 16A**). However, as shown in **Figure 16B**, the protective role of PTA on AcP stability was completely dependent on the presence of CoA – an example of cooperative binding. It is interesting to speculate if a PTA-dependent protective effect in AcP-degradation could be achieved *in vivo*, but likely the effect is related to the high concentrations of CoA provided in the *in vitro* reaction (in equimolar amounts to AcP: 10 mM).

The observation that high degree of AcP remained in the Pta(SE) samples even after 30 min furthermore indicated that the equilibrium of the reaction favors AcP formation. Even though the acetyl-CoA forming reaction is thermodynamically favorable at standard conditions ($\Delta G_R°'$=-9.8 kJ mol^{-1}), the intracellular concentrations of reactants and products might not support the formation of acetyl-CoA *in vivo*. Additionally, due to the reversibility of the reaction, host organisms commonly regulate the pathway flux by means of allosteric mediators such as NADH, ATP, pyruvate and PEP, all of which are strong inhibitors of the acetyl-CoA forming reaction of PTA in *E. coli* [272]. These effectors are likely to influence enzyme activity also in a heterologous host.

Due to the problems encountered trying to establish an *in vitro* assay, we decided to move ahead with the Pta(SE) candidate enzyme that had previously been utilized successfully in a PO/PTA-dependent strategy [249]. We began to evaluate the performance of the wild type versus the mutant strain *gpp1*Δ*gpp2*Δ, either carrying the control plasmid pSP-GM1 or plasmids containing Xfpk(BB) or Xfpk(BB)/Pta(SE). **Figure 17** summarizes the impact of the different modifications on **A)** μ(max), **B)** acetate formation and **C)** overall growth profiles.

Co-expression of XFPK/PTA in the wild type strain had a slight negative impact on growth when compared to expression of XFPK alone, both in terms of growth rate and final biomass yield (**Figure 17A** (blue bars) and **17C**). As a large proportion of the AcP in the wild type strain is hydrolyzed directly into acetate, the equilibrium reaction might however favor AcP formation from acetyl-CoA, creating a futile cycle that in turn reduces biomass formation. Indeed, there is no significant difference in the maximum amount of acetate accumulation in the strain expressing XFPK or XFPK/PTA **(Figure 17B),** indicating that the endogenous pathway has a much higher capacity than PTA to consume AcP.

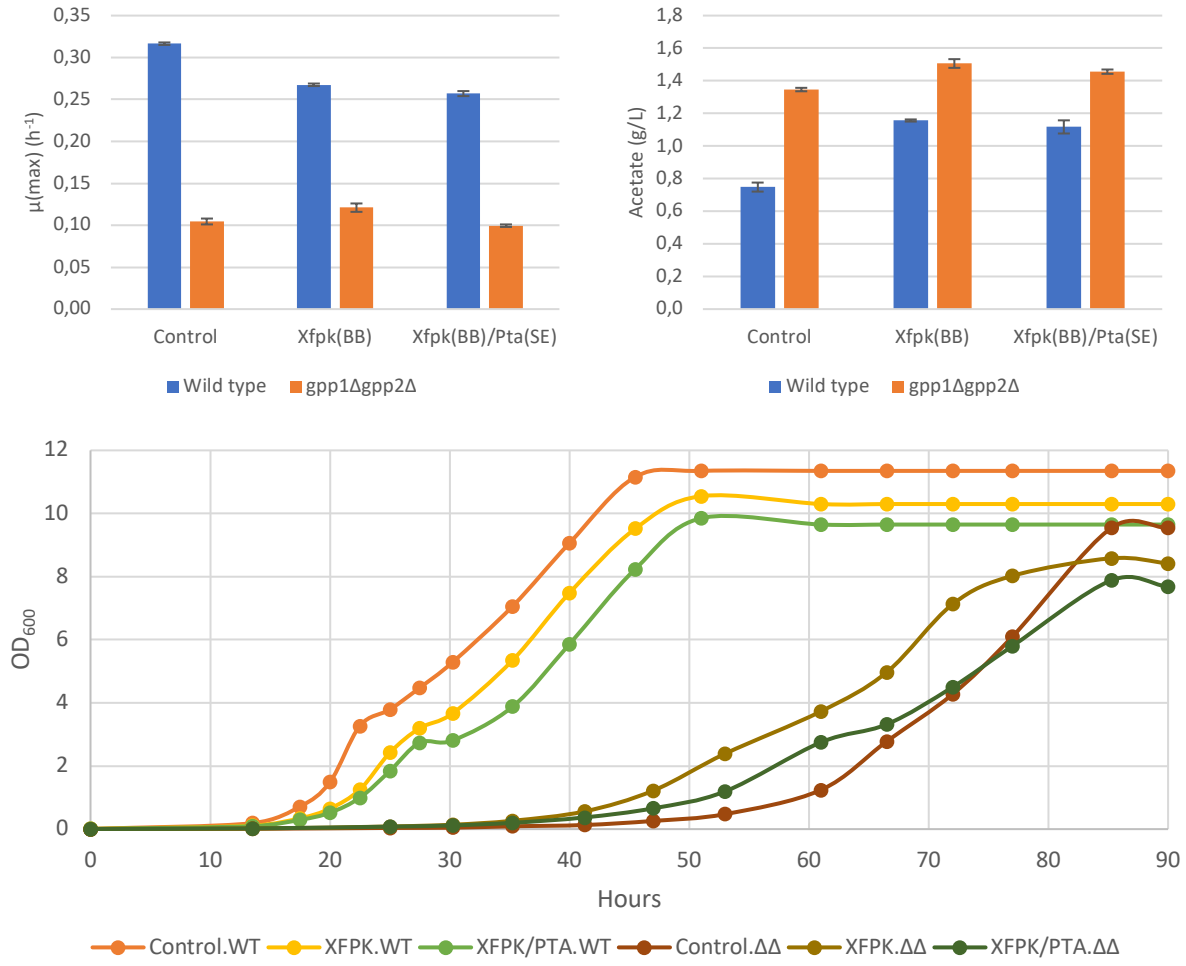


Figure 17. Cultivation of CEN.PK 113-5D (WT) or *gpp1*Δ*gpp2*Δ (ΔΔ) carrying an empty plasmid (Control), a plasmid encoding Xfpk(BB), or a plasmid encoding Xfpk(BB)/Pta(SE). The figures show the influence of **A)** the maximum specific growth rate μ(max) **B)** maximum level of acetate accumulation and **C)** growth profiles. The strains were grown in biological duplicates (shake flasks) in minimal media with 2% glucose.

The growth profile of the *gpp1*Δ*gpp2*Δ deletion strain clearly shows a significant effect of blocking glycerol formation: a severely reduced maximum specific growth rate (0.1 h^{-1} versus 0.32 h^{-1} of the wild type) and a strongly prolonged lag-phase (**Figure 17A** (orange bars) and **17C**). Glycerol formation is considered to have an important role in osmoregulation and redox control, primarily in anaerobic conditions, but also at high glucose concentrations when the respiratory system is repressed [66, 273]. Previous studies of a *gpp1*Δ*gpp2*Δ deletion mutant indeed support that the phosphatases have an important role during anaerobic growth, an effect linked to a disturbed NADH/NAD^+ ratio [274]. A double deletion of the glycerol-3-phosphate

dehydrogenases *GPD1* and *GPD2*, also resulting in a glycerol deficient phenotype, was similarly reported to generate a severe growth defect also during aerobic growth [275]. Expression of XFPK or XFPK/PTA in the *gpp1*Δ*gpp2*Δ strain moderately increased its growth rate and/or shortened the lag phase, possibly due to the redirection of carbon from lower glycolysis which should otherwise elevate the NADH-induced stress.

Interestingly, the acetate formation in the *gpp1*Δ*gpp*2Δ strain background is severely elevated compared to the wild type, by approximately 80% (**Figure 17B**), an observation that was also made by Nissen et al. when they cultivated a *gpd1*Δ*gpd2*Δ strain aerobically [275]. The elevated acetate level is likely to contribute to the impairment of growth of the strains. Expression of XFPK or XFPK/PTA in the *gpp1*Δ*gpp2*Δ strain only moderately increased the maximum acetate levels, approximately by 10% to the reference strain, which is a reduction compared to the acetate increase observed upon XFPK expression in the wild type strain, which was closer to 50%. However, the large overall increase in acetate production in itself defeats the purpose of deleting the genes.

In view of the severely impaired growth and high acetate production of the double deletion mutant, it would be more useful to choose a strategy where only one of the two phosphatases were deleted. As indicated in **Figure 10**, *GPP1* would be the preferred candidate for deletion although it would still result in a partial drain of the XFPK-formed AcP toward acetate. Indeed, this approach was adopted by Meadows et al., who utilized the dual route of XFPK/PTA and A-ALD to promote acetyl-CoA formation and ultimately farnesene synthesis in a Δ*gpp1* yeast host [276].

Thus, as a last experiment we expressed Xfpk(BB) alone or in combination with PTA from *Clostridium kluyveri* (Pta(CK)) – the same enzyme utilized by Meadows et al. [276] – in a yeast background carrying a deletion of *GPP1,* and evaluated the influence of the pathway on FA synthesis. Further, in order to enhance FA synthesis, we performed the experiment in a host that was devoid of the acyl-CoA synthetase genes *FAA1* and *FAA4*. This makes the strain unable to activate free fatty acid (FFA) species which as a consequence get secreted into the growth medium [12, 160].

We decided to investigate FFA synthesis at two stages of growth: at the end of exponential phase and after 72 h, when strains should have entered stationary phase. **Figure 18A** shows the OD_{600} reached after 72 h of cultivation time. The deletion of *GPP1* in the *faa1*Δ*faa4*Δ background had a negligible effect on final biomass yield. Xfpk(BB) expression in the strains had negative effect on growth, both in the wild type and *gpp1*Δ strain background. This is expected, as AcP still can be hydrolyzed to acetate by Gpp2 and possibly other intracellular phosphatases, albeit at a lower rate. Xfpk(BB)/Pta(CK) expression in the wild type exhibited the most hampered growth, possibly due to the formation of a futile cycle. It should be noted that all of these strains (marked with asterisks in figure) showed residual levels of the fermentation products ethanol, glycerol and acetate (data not shown), and thus it does not represent the final OD_{600} of the cultures. In particular the level of acetate accumulation in the Xfpk(BB)/Pta(CK) was very high – 2.8 g/L – considerably higher than the recorded concentration at glucose depletion (1.29 g/L). Furthermore, the biomass increase between the two sampling points was only 30%. This suggests that a majority of the energy produced by the cell is directly consumed to maintain cellular homeostasis in response to acetic acid stress.

Co-expression of Xfpk(BB)/Pta(CK) in the *gpp1*Δ background on the other hand significantly increased the performance in terms of growth. Within 72 h, the strain had successfully consumed all of the nutrients in the culture. Likely this effect can be explained by an elevated intracellular concentration of AcP that would enable a more efficient channeling towards acetyl-CoA and a decreased rate of futile cycling.

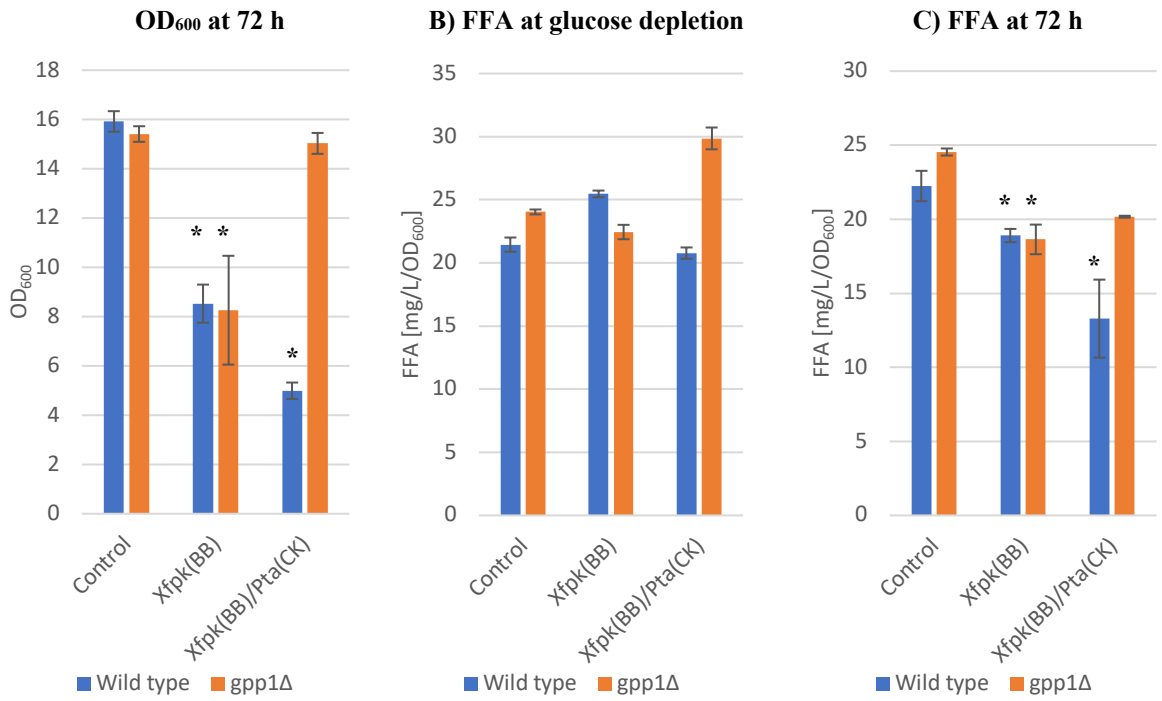


Figure 18 Cultivation of CEN.PK 113-5D *faa1*Δ*faa4* wild type (WT) or with an additional deletion of *GPP1* (gpp1Δ) carrying an empty plasmid (Control), a plasmid encoding Xfpk(BB), or a plasmid encoding Xfpk(BB)/Pta(CK). The figures show the influence on **A)** the OD_{600} at 72 h of culture **B)** free fatty acid accumulation per L medium and OD_{600} at glucose depletion and **C)** free fatty acid accumulation per L medium and OD_{600} at 72 h of culture. The strains were grown in biological triplicates in minimal media containing 2% glucose. Asterisks (*) indicate that HPLC measurements showed residual amounts of ethanol, glycerol and acetate in the culture.

As indicated by the FFA production in strains carrying the control plasmid, the deletion of *GPP1* had a slight positive effect on FFA synthesis (**Figure 18B**). Possibly, this could be related to the fact that glycerol-3-phosphate – which is likely to accumulate in the *gpp1*Δ strain – is a substrate for phospholipid and triacylglycerol (TAG) synthesis. Cellularly produced FFAs in yeast have been shown to stem from the hydrolysis of neutral lipids and phospholipids [12, 16].

When FFA synthesis is considered in the wild type background at glucose depletion (**Figure 18B**, blue bars), Xfpk(BB) expression alone had a small positive effect, while co-expression with Pta(CK) abolished the effect. The beneficial effect observed for strain Xfpk(BB) agrees with the pathway´s ability to redirect carbon from glycolysis – and subsequent ethanol fermentation – directly to acetate synthesis. Whereas the acetyl-CoA formed from acetate in the Xfpk(BB) strain to a larger degree is utilized by Acc1 and the FAS complex for FA synthesis, the surplus likely gets converted to AcP in the Xfpk(BB)/Pta(CK) strain – closing the futile cycle.

Xfpk(BB) expression in the *gpp1*Δ mutant led to a minor reduction in FFA synthesis in the glucose phase (**Figure 18B**, orange bars), possibly due to a larger proportion of the carbon being trapped in form of AcP during the glucose consumption phase, reducing the immediate benefit of the carbon redistribution. A 24% increase in FFA production was observed in the corresponding Xfpk(BB)/Pta(CK) strain, indicating a successful diversion of C5 and C6 carbon directly toward acetyl-CoA synthesis via the heterologous pathway. The acetate levels at glucose depletion in the Xfpk(BB)/Pta(CK) strain were not significantly different from the control, while the acetate levels in the Xfpk(BB) background was nearly increased 2-fold, indicating that the *GPP1* deletion is sufficient to prevent acetate accumulation in a XFPK-expressing strain when co-expressed with a PTA.

After 72 h of cultivation time, XFPK and XFPK/PTA expression proved to have a negative impact on FFA synthesis in both the wild-type and the *gpp1*Δ background. As such, FFA synthesis was reduced by 20 % in the Xfpk(BB)/Pta(CK) *gpp1*Δ strain compared to the control (**Figure 18C**). When ethanol is metabolized it is converted to cytosolic acetyl-CoA before being used for various cellular processes such as respiratory assimilation of the carbon in the TCA cycle. During such circumstances, the concentration of AcP produced by XFPK should be low. This can be expected as flux through gluconeogenesis and the PPP likely provides a significantly lower flux than that of glycolysis during sugar consumption. Thus, the equilibrium reaction of PTA would potentially favor AcP formation from acetyl-CoA during growth on ethanol.

Taken together, this suggests that the optimal setting, in which to use the XFPK/PTA pathway to stimulate production of acetyl-CoA derived compounds, is to prevent a two-stage fermentation and ensure continuous provision of glucose as substrate throughout the process. This can for example be achieved by employing a fed-batch process, in which the glucose is slowly fed throughout the whole cultivation to prevent of overflow metabolism. This is a common cultivation technique in industrial settings, and indeed, Meadows et al. used a type of fed-batch operation to optimize the XFPK/PTA-dependent production of farnesene [276].

4 Influencing fatty acid synthesis via an increased NADPH supply

4.1 Engineering fatty acid metabolism

Various metabolic engineering strategies to positively influence production of fatty acids (FAs) and fatty acid derived compounds in *S. cerevisiae* have been implemented. As presented in **Chapter 3**, one approach has been to increase supply and/or introducing routes which provide a more resource-efficient provision of the fatty acid precursor acetyl-CoA. In the following subchapter, some of the more general strategies will be briefly introduced, an overview of which are shown in **Figure 19**. Commonly, several of these are combined simultaneously with expression of a terminal enzyme to achieve the desired product, e.g. alkanes or fatty alcohols. It should be noted that this summary is far from being exhaustive.

Deregulating fatty acid synthesis

The initial step of FA synthesis is the formation of malonyl-CoA from acetyl-CoA, catalyzed by Acc1. As mentioned in **Chapter 2**, activity of Acc1 is negatively regulated by the kinase Snf1 [163]. Shi et al. showed that this mode of regulation could be abolished by removing the putative Snf1-phosphorylation sites S659 and S1157. While overexpression of the native enzyme did not have any significant impact on FA content of the strain compared to the control, expression of a gene with both serine residues replaced by alanine (*ACC1*$^{S659A,\ S1157A}$, also referred to as *ACC1***) enhanced total FA content by 65% [41]. The strategy has thereafter been utilized in multiple studies targeting FA-derived compounds, such as FAEEs, TAGs and fatty alcohols [18, 21, 26, 277].

Activity of Acc1 and FAS is also suggested to be regulated by acyl-CoA species, likely via steric as well as gene regulatory mechanisms (see **Chapter 2.3** for more details) [42, 176]. Thus, recently a common strategy has been to reduce "product-inhibition" by deleting enzymes responsible for free FA (FFA) activation in *S. cerevisiae*, in particular Faa1 and Faa4. In the double mutant *faa1*Δ*faa4*Δ, expression of *ACC1*, *FAS1*, *FAS2* and *OLE1* has been shown to be significantly increased [11]. The inability of the yeast to activate the FFA while they are formed from various cellular lipases lead to a secretion of FFA to the medium. While not significantly influencing intracellular FA concentrations (corresponding to approximately 300 mg/L), 200 mg/L FFA are excreted in a *faa1*Δ*faa4*Δ strain, generating a 60% increase in total FA titers [12]. In combination with other metabolic engineering strategies, such as prevention of β-

oxidation and expression of thioesterases (TEs) which release the fatty acid chain from CoA, the strategy has been utilized in various studies to achieve high levels of free fatty acids or products derived thereof [13, 15, 241, 278].

Another way to influence lipid synthesis is to modify the network regulating their synthesis. Feng et al. evaluated the impact of deleting various negative regulators of phospholipid synthesis, and observed that the product titer correlated with that of cellular phospholipid content, and in particular the deletion of the deacetylase *RPD3* resulted in a 2-fold increase in the FA-derived product fatty alcohol to 140 mg/L [279].

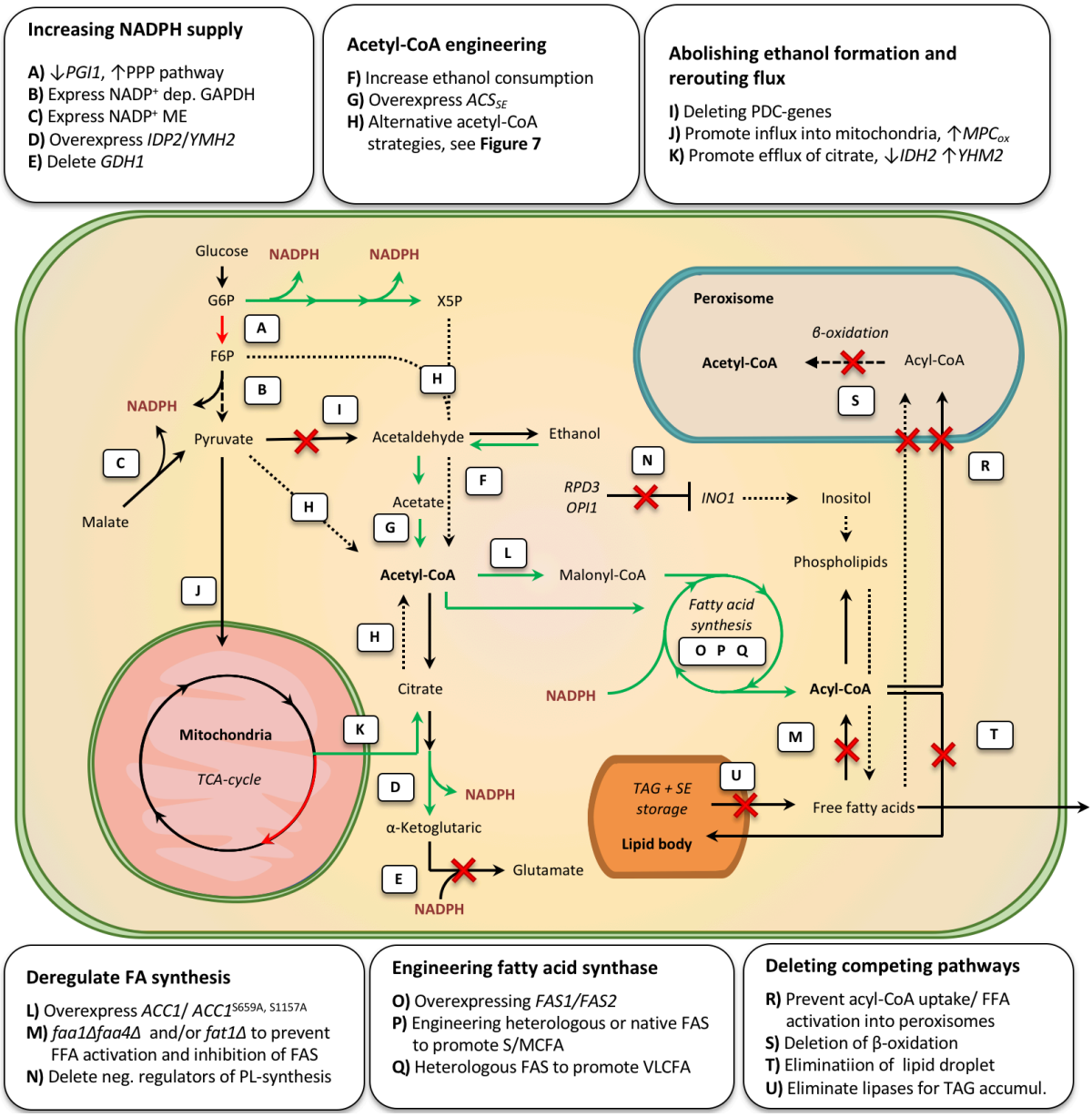


Figure 19. Metabolic engineering strategies to influence FA and FA-derived production in *S. cerevisiae.*

Expanding the fatty acid product spectrum

Naturally, *S. cerevisiae* FAS mainly produces FA species of C_{16}-C_{18}. Many commercially attractive products call for FAs of shorter or longer length, such as short-chain hydrocarbons in gasoline substitutes, and long-chain FAs in products such as lubricants, cosmetics and pharmaceuticals.

The FAS complex has been replaced or engineered to promote short/medium chain FA (S/MCFA) synthesis (C_6-C_{12}) in *S. cerevisiae*. Co-expression of a human FAS in which the native thioesterase (TE) domain had been replaced with a short-chain specific TE (sTE), preferentially releasing the FA-chain at an earlier stage during elongation, with a heterologous phosphopantetheine transferase enabled production of 111 mg/L of S/MCFAs [19]. The rationale for using the hFAS was due to its architectural flexibility, consisting of an X-shaped homodimer, in contrast with the highly complex fungal FAS. However, recent engineering efforts demonstrated that rational engineering of *S. cerevisiae* FAS efficiently can generate S/MCFAs. By introducing a sTE into Fas2, in combination with mutations in the KS domain restricting acyl chain elongation, S/MCFA synthesis reached 175 mg/L [20]. Similarly, by overexpressing a FAS-complex carrying three mutations located within 1) the KS domain (interfering with chain elongation), 2) the MPT domain (reducing affinity for malonyl-CoA) and AT-domain (broadening the acetyl-CoA binding channel), respectively, yeast was engineering strain was able to produce 464 mg/L C_6-C_8 FAs [22].

In terms of long-chain fatty acids, a FAS system from the bacterium *Mycobacterium vaccae* was recently introduced to yeast due to its ability to directly produce both long ($C_{16\text{-}18}$) and very long (VL: $C_{22\text{-}26}$) FA species. Through expression of the *M. vaccae* FAS and a long-chain specific alcohol reductase from *Arabidopsis thaliana* in conjunction with various other genetic perturbations, a production level of 83.5 mg/L docosanol (C_{22}) was achieved [21].

Deleting competing metabolic pathways

Another common approach is to prevent FAs from being consumed in competing reactions. FAs can be stored as neutral lipids – triacylglycerols (TAGs) and sterol esters (SEs) – in so-called lipid bodies, before being mobilized and used for growth when needed. The genes encoding TAG synthases (*DGA1* and *LRO1*) and acyl-CoA:sterol acyltransferases (*ARE1* and *ARE2*) have been found to be non-essential in yeast [280]. Another pathway that consumes FAs is β-oxidation, which degrades fatty acyl-CoA species to acetyl-CoA within the peroxisomes. A heterodimeric ABC transport complex is responsible for importing long-chain FA species into peroxisomes, encoded by *PXA1* and *PXA2*, while enzymes catalyzing FA activation and the initial step of degradation within the peroxisome are encoded by *FAA2* and *POX1, respectively* [281].

The described cellular features are commonly blocked to achieve higher yields of FAs and FA-derived products. Elimination of lipid droplet formation and the initial step of β-oxidation (*dga1*Δ*lro1*Δ*are1*Δ*are2*Δ*pox1*Δ) generated a 5-fold increase in free FA content [31]. Deletion of three genes responsible for fatty acid activation (discussed under section “deregulating fatty acid synthesis”) in combination with three genes contributing to β-oxidation (*faa1*Δ*faa4*Δ*fat1*Δ*pxa1*Δ*faa2*Δ*pox1*Δ) increased FFA titers to 1.3 g/L [12].

Similarly, in order to enhance titers in a strain already overproducing TAGs, a successful strategy included deletion of the SE synthase gene *ARE1*, TAG-lipase genes *TGL3*, *TGL4*, *TGL5*, the three mentioned peroxisomal β-oxidation genes as well as *GUT2* – responsible for consuming glycerol-3-phosphate required for TAG-synthesis – and led to a TAG content corresponding to 25% of the cell dry weight [18].

Engineering NADPH supply

FA synthesis requires large amounts of NADPH – two molecules per elongation cycle, corresponding to a cost of 16 NADPH for one molecule of oleic (C_{18}) acid. Consequently, also enhancing NADPH supply has been an approach positively influence FA production in *S. cerevisiae*. By introducing a $NADP^+$-dependent GAPDH from *Streptococcus mutans*, the FAEE titer was increased by approximately 20%, to 48 mg/L [30]. A $NADP^+$-producing malic enzyme from *Mortierella alpina* was also expressed to enhance production of fatty alcohols in yeast, which only resulted in a 5% (non-significant) increase [13].

A recent study presented a rigorous reprogramming effort to turn *S. cerevisiae* into a lipogenic yeast, which included several NADPH-engineering strategies [15]. In part, flux was re-routed through the oxidative PPP via fine-tuned downregulation of *PGI1*, responsible for isomerization of G6P to F6P in glycolysis, while four enzymes within the PPP were upregulated: *ZWF1*, *GND1*, *TKL1* and *TAL1*, leading to a 28% increase in FFA production. Furthermore, the yeast genes *YHM2* (encoding a citrate (out) / oxaloacetate (in) shuttle transporter in the mitochondrial membrane) and *IDP2* (responsible for transforming isocitrate to oxaloacetate in the cytosol) were overexpressed to elevate NADPH levels. The final strain produced 33.6 g/L FFA in a fed-batch cultivation, which is the highest reported so far by *S. cerevisiae* [15].

An alternative to supplying more NADPH, to decrease its consumption in competing pathways is also an option. Metabolic modelling of a fatty alcohol overproducing system indicated that 60% of the NADPH produced was consumed by the $NADP^+$-dependent glutamate dehydrogenase Gdh1, deletion of which improved fatty alcohol production 3.7-fold [17].

4.2 *STB5* overexpression as a metabolic engineering strategy (Paper III)

Among the previous strategies to increase FA synthesis via an enhanced NADPH supply discussed above, the re-direction of carbon flux through the oxidative PPP appears to be the most successful. This correlates well with the pathway´s ability to produce two molecules of NADPH per G6P entering the pathway. Indeed, an elevated flux through the oxidative PPP is utilized as cellular protection in response to oxidative stress, and without its integrity, cells encounter a redox-collapse [196, 282]. In **Paper III** we investigated a metabolic engineering strategy aimed at elevating intracellular NADPH supply by redirecting flux from glycolysis through the oxidative PPP through a mechanism which is naturally activated in yeast upon oxidative stress.

STB5 encodes a transcription factor (TF) shown to be involved in regulating NADPH generation in response to oxidative stress. Using chromatin immunoprecipitation (ChIP) and gene expression profiling, Larochelle et al. showed that Stb5 binds to the promoters and activates expression of multiple genes within the oxidative PPP, including *ZWF1*, *SOL3*, *GND1*, *GND2*, *RKI1*, *TAL1* and *TKL1*, upon diamide treatment [47]. Simultaneously, Stb5 interacted with the promoter of *PGI1*, whose expression in a *stb5*Δ mutant increased substantially compared to the wild type in response to diamide treatment, indicating that Stb5 can also act as a transcriptional repressor. In addition to its role in regulating PPP-genes, Stb5 was found to be an activator of the genes *ALD6*, *ALD4*, *IDP2*, *ILV5*, *ADH6, GOR1*, *YEF* and *YMR315* – also known to have roles in NADPH-producing reactions [47, 213].

This suggests that an increased flux through the PPP as well an increased pool of NADPH potentially could be achieved simply by enhancing the activity of Stb5. Indeed, overexpression of *STB5* from a low-copy plasmid increased NADPH levels 2.5 times in a BY4727 background [213]. Strong overexpression of *STB5* prevented the host strain to grow on glucose, but this

growth defect was partly relieved when overexpressing a NADPH-consuming butanediol dehydrogenase, indicating that *STB5* overexpression led to a redox imbalance [283].

In the light of these findings, in **Paper III** we evaluated if promoter replacement of *STB5* could be used as a tool to positively influence FA synthesis. The hypothesis is that it potentially could activate several NADPH-producing reactions simultaneously while downregulating flux through upper glycolysis. In **Figure 20**, an overview of central carbon metabolism and some of the key targets of Stb5 anticipated to be perturbed are shown. If successful, this strategy could represent a flexible and easy-to-implement strategy to boost NADPH supply for various reductive biosynthetic processes.

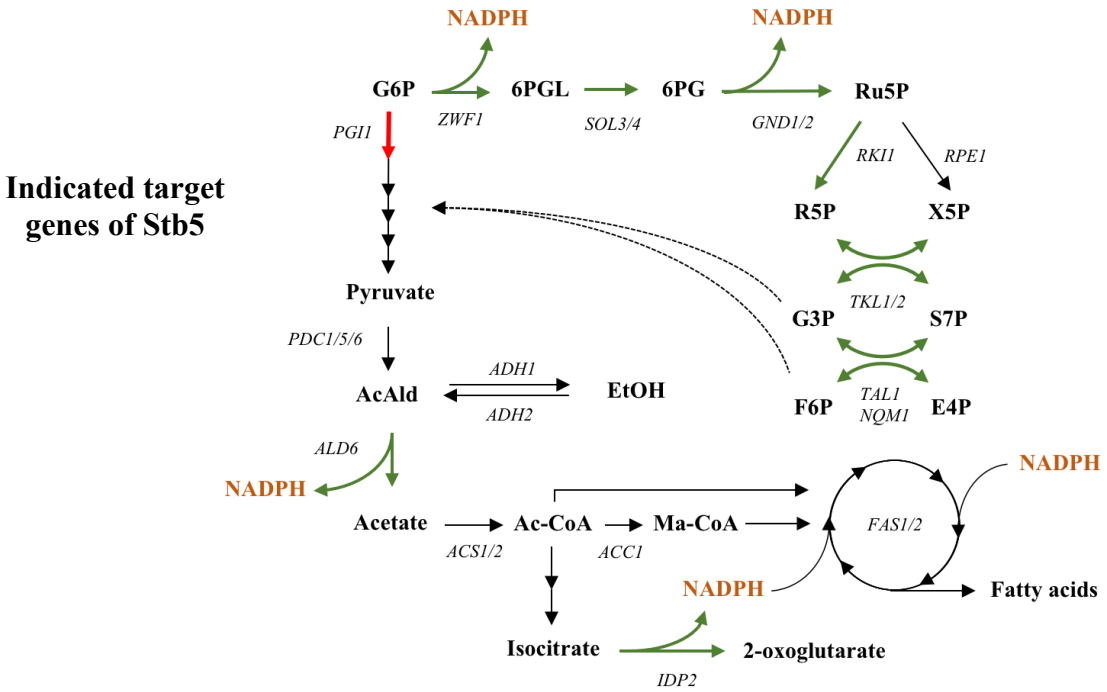


Figure 20. Overview of the key targets of the transcription factor Stb5. Positively and negatively regulated reactions are highlighted in green and red, respectively. Adapted from Bergman et al. [284].

Initially, we evaluated various promoters to tune expression of *STB5*, as a previous study indicated that strong overexpression prevented the strain to grow on glucose [283]. Using a plasmid-based CRISPR/Cas9-approach, we replaced the native promoter with that of the Cu^{2+}-responsive promoter of *CUP1* and promoters of *CYC1* (weaker), *ADH1*, *TPI1* and *PGK1*. We did not observe a growth arrest on glucose even in the strains where *STB5* was regulated by strong promoters. However, both Cu^{2+} induction and utilization of constitutive promoters significantly decreased μ(max) and final biomass yields compared to the control in an expression induction/promoter strength-dependent manner. The recorded maximum specific growth rates are shown in **Table 4**.

Cadiere et al. argued that the negative growth-related effect of *STB5* overexpression was due to a redox imbalance, as growth was partially resumed when a NADPH-consuming enzyme was overexpressed in the strain [283]. Furthermore, plasmid-based overexpression of *STB5* in a BY4727 background was in a previous study reported to increase the NADPH concentration 2.5-fold [213]. We measured the NADPH content of the constitutive promoter exchanged strains in exponentially growing cultures, however failing to detect any significant difference compared to the control in any of the strains.

Table 4. Maximum specific growth rates (μ(max) [h^{-1}]) of *STB5*-promoter exchanged strains (AB12-AB16).

A)* STB5 *overexpression mediated by copper induction				
Cu^{2+} (μM)	**Control (CEN.PK 113-5D)**	**P_{CUP1}-*STB5* (AB12)**		
"0"	0.317 ± 0.034	0.344 ± 0.018		
100	0.254 ± 0.014	0.259 ± 0.003		
200	0.284 ± 0.014	0.227 ± 0.008 *		
400	0.181 ± 0.004	0.107 ± 0.021 *		
B)* STB5 *overexpression mediated by constitutive promoters				
Control (CEN.PK 113-5D)	**P_{CYC1}-*STB5* (AB13)**	**P_{ADH1}-*STB5* (AB14)**	**P_{TPI1}-*STB5* (AB15)**	**P_{PGK1}-*STB5* (AB16)**
0.326 ± 0.012	0.265 ± 0.006 *	0.239 ± 0.002 *	0.246 ± 0.009 *	0.219 ± 0.014 *

Asterisks (*) indicate a significant difference ($p<0.05$; Students t-test, two-sided) compared to the control at the same conditions. Strains were grown in minimal media with 2% glucose and 60 mg/L uracil. Values correspond to the average of biological triplicates ± standard deviation.

As an alternative strategy, we investigated how two *STB5*-overexpressing strains and a *zwf1*Δ mutant, known to be sensitive toward oxidative stress, handled oxidative stress during the exponential growth phase by measuring their ability to form viable colonies after a H_2O_2 shock. The proportion of H_2O_2-shocked cells that survived the treatment (normalized to colony count of strains when incubated in regular media) are presented in **Figure 21**. Interestingly, overexpression of *STB5* reduced resistance toward oxidative stress, as indicated by similarity in behavior of the *zwf1*Δ strain and P_{TPI1}-*STB5*. This counterintuitively implies that the flux through the PPP is impaired upon *STB5* overexpression, as the flux immediately is re-routed through this pathway as a primary response to oxidative stress [282].

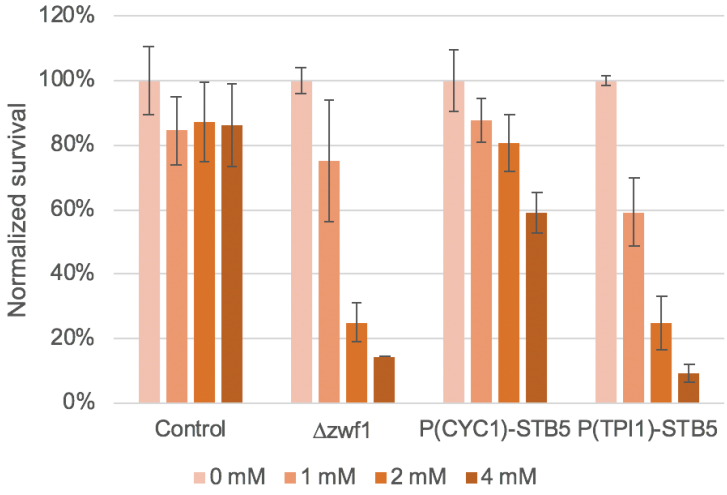


Figure 21. Survival of the strains CEN.PK 113-5D, *zwf1*Δ and P_{CYC1}-*STB5* and P_{TPI1}-*STB5*after liquid H_2O_2-shock. The values indicate the proportion of surviving cells at each concentration compared to the value observed for each strain at 0 mM H_2O_2. Values are averages of two biological and two technical replicates; error bars represent ± standard deviation. Adpated from Bergman et al. [284].

Even though we were unable to experimentally demonstrate that *STB5* overexpression was linked to an increased NADPH supply, we decided to investigate if overexpression had an effect on FA synthesis. In order to stimulate FA production and generate a drain of NADPH, *STB5* promoter replacements were integrated into the double deletion strain CEN.PK 113-5D *faa1*Δ*faa4*Δ. While in *faa1*Δ*faa4*Δ strains with a strong promoter controlling *STB5,* the growth

rate was reduced in a similar manner as in CEN.PK113-5D (62-72% of that observed for the control), the growth rate of P_{CYC1}-*STB5* was not significantly altered compared to the control (data not shown) – possibly an indication of that a redox-imbalance was alleviated.

We decided to harvest samples for FFA analysis at the end of glucose phase as well at stationary phase (72 h) as we expected *STB5* to have a greater influence on modulating flux in glucose phase. As shown in **Figure 22A**, weaker as well as stronger *STB5* overexpression resulted in an elevated capacity to produce FFAs in the glucose phase, ranging from 130-139% of that observed for the control. The effect did however not correlate clearly with *STB5*-expression level, and the differences between the promoter exchanged strains were insignificant. At 72 h, stronger overexpression of *STB5* led to a decreased ability to produce FFAs, while the final titers of P_{CYC1}-*STB5* were not significantly different from the control. This indicates that effects mediated by Stb5 in the ethanol phase in some way interfere with FFA synthesis.

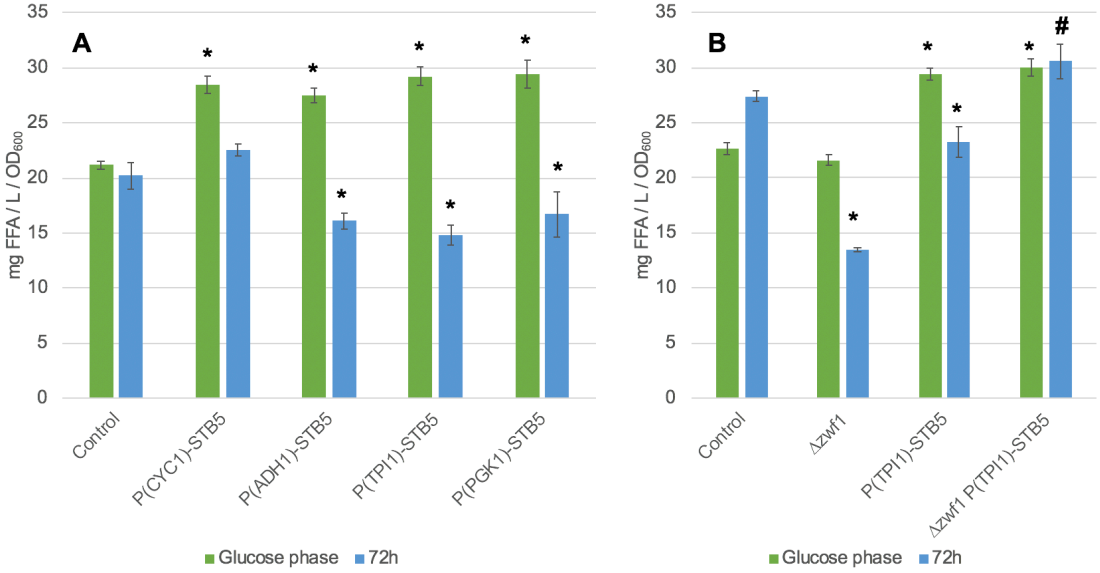


Figure 22. Free fatty acid quantification of CEN.PK 113-5D *faa1*Δ*faa4*Δ strains after glucose was depleted (Glucose phase) and at stationary phase (72 h). **A)** Influence of different levels of *STB5* overexpression on fatty acid synthetic ability. **B)** Influence of *ZWF1* deletion on fatty acid synthetic ability in the control and P_{TPI1}-*STB5* strain (# indicates that the strain failed to consume the produced ethanol). Strains were grown in minimal medium with 2% glucose with the addition of 60 mg/L uracil. Values are averages of three biological replicates, error bars ± standard deviation. Asterisks (*) indicate a significant change from the control ($p<0.05$; Students t-test, two-sided) at the corresponding sampling point. Adapted from Bergman et al. [284].

In order to clarify if the oxidative PPP had a role in providing reducing equivalents for FFA synthesis in *STB5*-overexpression strains, we deleted *ZWF1* in the *faa1*Δ*faa4*Δ control and P_{TPI1}-*STB5*. The results of the FFA quantification are shown in **Figure 22B**, and the results from the glucose phase confirm that the positive effect *STB5* overexpression has on FFA synthesis is independent of a flux through the oxidative PPP. The *zwf1*Δ deletion in the P_{TPI1}-*STB5* mutant did not positively affect the final titers, but prevented the strain to resume growth after glucose depletion. A noteworthy observation (without direct relation to the topic of *STB5*) is that the oxidative PPP appears to contribute significantly to FFA synthesis during growth on ethanol, as the final titers were reduced by 50% compared to the control. If it provides reducing equivalents also during glucose phase, this can be compensated via an enhanced flux through other routes. In the oleaginous yeast *Y. lipolytica*, the oxidative PPP has been shown to be the main contributor of NADPH during lipid formation [285], but such a relationship has not been established in *S. cerevisiae.*

As overexpression of *STB5* using weak (P_{CYC1}) as well as strong promoters (P_{ADH1}, P_{TPI1} and P_{PGK1}) produced a similar effect on FA production in the glucose phase (**Figure 22A**), even though the μ(max) of the respective strains differed substantially, we decided to investigate if FFA production in the glucose phase could be limited by other factors than NADPH – such as the precursor acetyl-CoA. Therefore, we utilized the Xfpk(BB)/Pta(CK) pathway introduced in **Chapter 3.4** to promote cytosolic acetyl-coA synthesis in strain P_{TPI1}-*STB*. As we observed *gpp1*Δ to be essential to generate a positive effect on FFA-synthesis (**Figure 18B**), the expression of the XFPK/PTA pathway was evaluated by transforming the control plasmid pSP-GM1 and pAB10 (Xfpk(BB)/Pta(CK)) into the following *faa1*Δ*faa4*Δ strains: 1) a non-modified control , 2) *gpp1*Δ control and 3) *gpp1*Δ P_{TPI1}-*STB*. Figure 23 shows the FFA titers obtained at the end of glucose phase normalized to sampling OD_{600}.

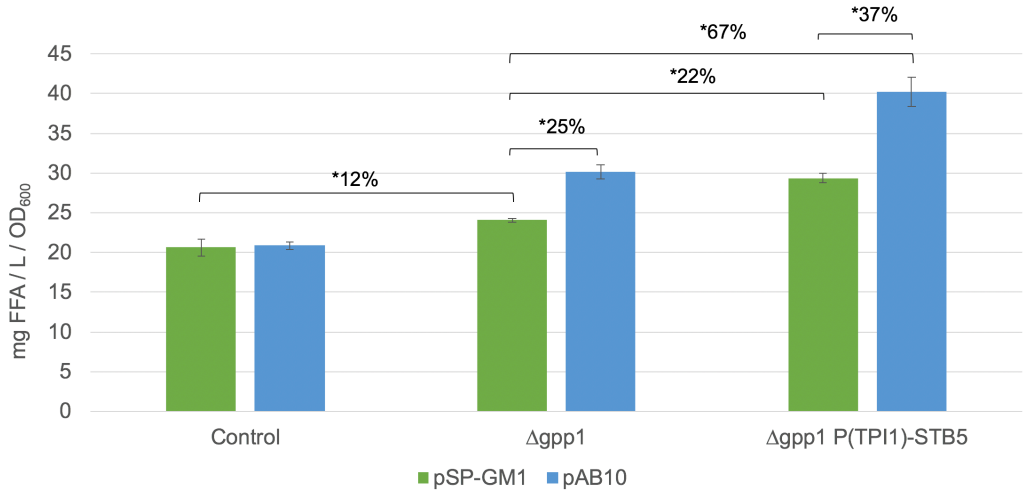


Figure 23. Influence of additional acetyl-CoA supply on free fatty acid production of CEN.PK 113-5D *faa1*Δ*faa4*Δ strains on glucose. The indicated host strains were either transformed with the empty plasmid pSP-GM1 or pAB10, encoding a XFPK from *B. breve* and a PTA from *C. kluyveri*. Strains were grown in minimal media with 2% glucose and harvested at or close to glucose to glucose depletion. Values are averages of three biological replicates, error bars ± standard deviation. Asterisks (*) indicate a significant change ($p<0.05$; Student's t-test, two-sided). Adapted from Bergman et al. [284].

Similar to what was observed in **Figure 22**, overexpression of *STB5* had a positive effect on FFA synthesis in the glucose phase also in the *gpp1*Δ background (**Figure 23**, AB29 vs AB27), albeit to a lower extent than observed in the non-deletion background (22% vs. 38% increase). Expression of the Xfpk(BB)/Pta(CK) pathway in the *gpp1*Δ strains had a positive effect in both strains, but while the increase was approximately 25% in the control strain, it reached 37% in the *STB5*-overexpression strain.

These results indicate that the effects of *STB5* overexpression and an increased acetyl-CoA supply are not just additive, but act in synergy to increase FFA synthesis. This does in turn imply that *STB5* overexpression makes more NADPH available than what efficiently can be utilized for the desired purpose of FFA synthesis, which instead is consumed via other competing cellular processes.

With regards to the conflicting results we observed, i.e. that previously published results suggest that Stb5 is an activator of the oxidative PPP while our results indicate that flux through the pathway is blocked, we wanted to make a more detailed characterization of the effects of overexpression, including RNAseq to get a holistic view of the gene expression profile. The control strain CEN.PK 113-5D and P_{TPI1}-*STB5* (AB15) were cultivated in quadruplicates and samples for RNAseq were taken in glucose phase, ethanol phase and during a glucose limited steady state ($D=0.1\ h^{-1}$). HPLC and biomass sampling during the exponential glucose phase

growth showed that *STB5* overexpression led to a 35% decrease in μ(max) (0.25 vs. 0.39 h^{-1}) and a significant reduction in glycerol, ethanol and biomass flux even when the parameters were normalized to the glucose uptake rate (corresponding to a decrease of 35%, 18% and 17%, respectively).

An overall PCA plot (**Figure 24**) of the RNAseq data showed the greatest difference in expression profile between all the 6 different sample groups (2 strains and 3 conditions) was explained by the sampling condition, in particular between samples taken in exponential phase and in ethanol/steady state which corresponds well to the large rearrangement in metabolism associated with a shift from fermentative to respiratory growth. Furthermore, the data show that *STB5* overexpression mediates the greatest gene expression change in glucose phase, which appears to shift the expression profile to a state more similar to one of more respiratory character. A gene set analysis (not shown) performed with the software PIANO highlights that respiratory processes indeed were induced in strain AB15 during the glucose phase, as well as a downregulation of multiple processes related to protein synthesis, which is to be expected as the growth rate is known to have a large influence on expression of genes related to ribosome and protein biogenesis [286].

STB5 expression was significantly enhanced compared to the control in all conditions, with a $\log_2$ fold change (LFC) of 4.83, 1.75 and 3.29 in glucose, ethanol and steady state, respectively. The native expression levels of *STB5* were low at all conditions, with FPKM-values between 16 and 27, which for example can be compared to that of the glycolytic gene *PGK1* in the glucose phase, averaging close to 3000.

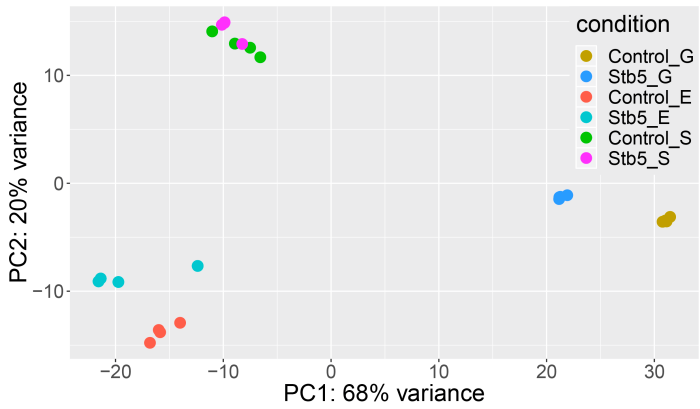


Figure 24. PCA plot of RNAseq data from control and P_{TPI1}-*STB5* strain. G, E and S corresponds to the sampling points (Glucose, Ethanol and Steady state, respectively). Adapted from Bergman et al. [284]

We performed a reporter transcription factor analysis – matching the transcriptome data to a database which lists TFs and their known targets and type of regulation – to find which TFs were likely to induce the observed expression changes in the strain overexpressing *STB5* compared to the control. The analysis did not indicate Stb5 as a probable inducer in neither the glucose nor ethanol phase, while Stb5 appeared as the most likely candidate to drive the observed expression changes during steady state conditions (**Figure 25A**). This indicates that mechanisms are present to counteract the effects of Stb5-mediated regulation in glucose and ethanol phase. Adr1, essential for activation of glucose-repressed genes when cells enter the diauxic shift [126], stood out as a key positive TF in the glucose phase, together with Msn2, Msn4, Pdr1 and Sok2 – transcriptional activators involved in stress response, pleiotropic drug resistance and cAMP-dependent protein kinase signal transduction. Of these, only *ADR1* showed a prominent degree of upregulation (**Figure 25B**). In a recent study by Ouyang et al, *ADR1* and *MSN4* were indicated as direct targets of Stb5, although, *ADR1* was reported to be negatively regulated [287].

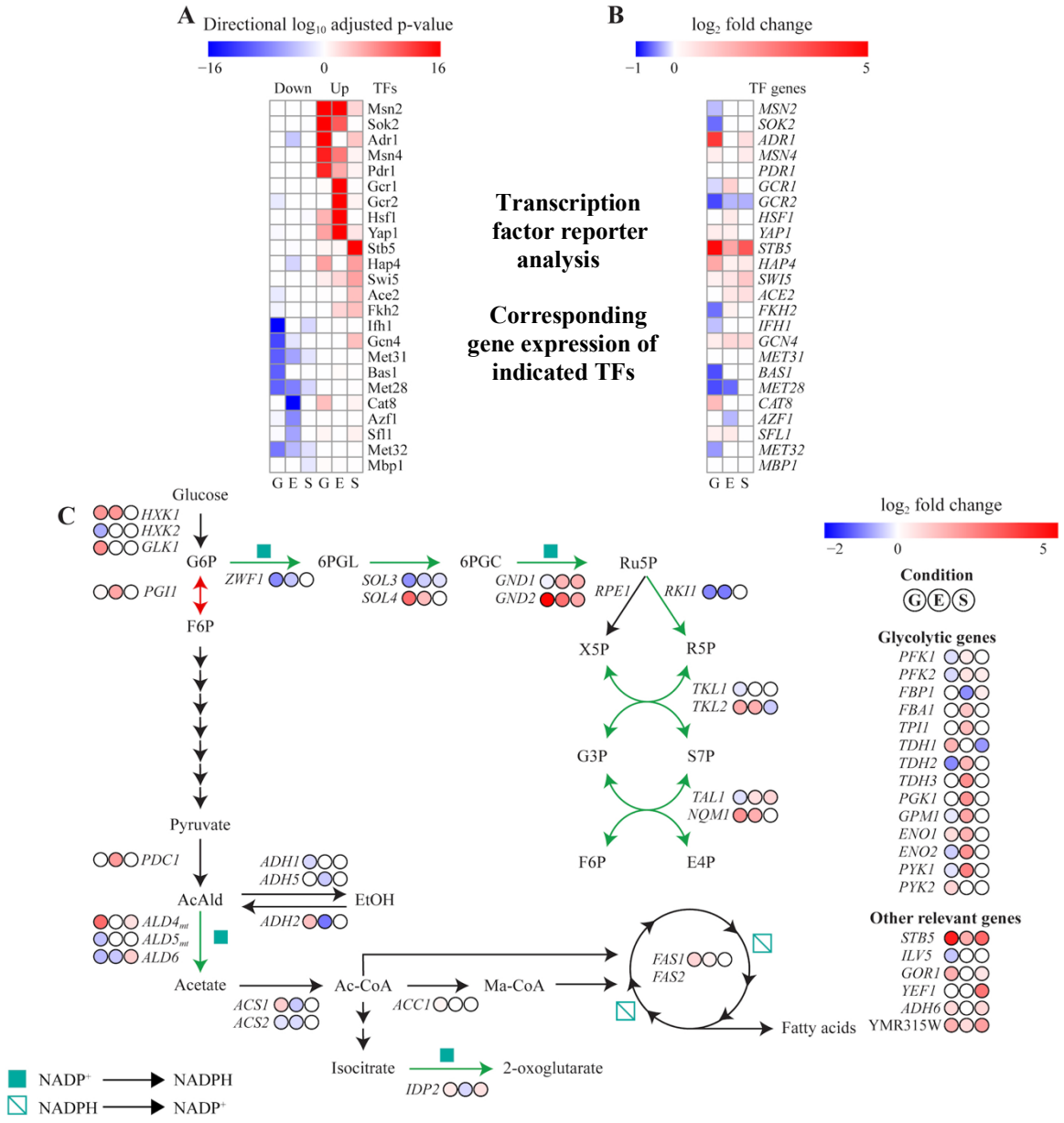


Figure 25. Transcriptional analysis of *STB5* overexpression in different conditions, where G, E and S correspond to the sampling point "Glucose", "Ethanol" and "Steady State", respectively. A) Transcription factor (TF) reporter analysis, showing the top five scored TFs predicted to drive the down- and upregulated transcriptional changes at each of the tested conditions. B) Differential expression of the reporter TF genes in respective conditions (padj<0.01). C) Differential expression analysis of genes involved in the PPP, glycolysis, fatty acid synthesis and suggested STB5 target genes (padj<0.01). Adapted from Bergman et al. [284].

When investigating differentially expressed genes within the central carbon metabolism and previously indicted target genes of *STB5* in the different sampling conditions (**Figure 25C**), we observed a clear alteration in expression of genes within the PPP in the glucose phase. *ZWF1*, *SOL3, RKI1, TAL1* and *TKL1* were significantly downregulated, the expression level of *GND1* and *RPE1* remained unchanged, while *SOL4*, *GND2, NQM1* and *TKL2* were induced upon *STB5* overexpression. It should however be noted that *SOL4*, *GND2*, *NQM1* and *TKL2* all had significantly lower FPKM values than their corresponding paralogs in the glucose phase. When the raw read counts for each gene pair were added together and the combined LFC for *SOL*,

GND, *TAL* and *TKL* were calculated with DESeq2, only *GND* had a slightly positive value while all other PPP reactions were affected negatively (significant LFC-values: -0.69, 0.18, -0.26, and -0.24, respectively). Taking the downregulation of *ZWF1* and the "non-materialized" downregulation of *PGI1* into account, it strongly suggests a reduced capacity of flux through the PPP in the glucose phase.

Among the other genes indicated to be regulated by Stb5 in response to oxidative stress, *ALD6* and *ILV5* were downregulated, *YEF1* remained unchanged while *IDP2*, *GOR1*, *ALD4*, *ADH6* and *YMR315W* were upregulated in response to *STB5* overexpression in the glucose phase. Even though *IDP2* had a positive LFC in the DE analysis, it is unlikely that this has a large influence on metabolism in the glucose phase due to a low FPKM value. *ALD4* was strongly upregulated, but the subcellular localization of the corresponding enzyme is within the mitochondria and the suggested redox shuttle gene *YHM2* was significantly downregulated (LFC= -1.31). The DE analysis also shows a weak but positive effect on expression of genes with a direct relevance for FA biosynthesis, such as *ACC1* and *FAS1* (LFC=0.21 and 0.90).

STB5 overexpression caused a coordinated upregulation of glycolytic genes (**Figure 25C**) in the ethanol phase, which likely is mediated through an upregulation of the transcription factor gene *GCR1* highlighted in the TF analysis (**Figure 25A** and **25B**), which is instrumental in regulating expression of glycolytic genes [288]. Simultaneously, gluconeogenic genes (*FBP1*, *PCK1*, *PYC1*) and the initial gene in the oxidative PPP (*ZWF1*) were downregulated compared to the control, which collectively indicates that the flux through the oxidative PPP was reduced also in the ethanol phase (Figure 6C). As observed in **Figure 22B**, the oxidative PPP appears to play a substantial role in providing NADPH for FA synthesis particularly in the ethanol phase, which could help to explain the observed negative effect *STB5* expression had on FFA formation in this phase

At steady state conditions, fewer genes of the PPP were significantly deregulated by *STB5* overexpression. *ZWF1*, *SOL4* and *RKI1* were not significantly changed, *SOL3* and *TKL2* were downregulated, while *GND1*, *GND2*, *RPE1* and *TAL1* were upregulated. Of the other genes indicated to be activated by Stb5 in the study of Larochelle et al., almost all were upregulated: *ALD6*, *IDP2*, *GOR1*, *YEF1*, *ALD4*, *ADH6* and *YMR315W*. This sheds light on the result of the reporter TF analysis, which indicated that Stb5 was likely to drive the observed gene expression changes in steady state but not in glucose nor ethanol phase (**Figure 25A**).

The RNAseq analysis shows that some previously indicated target genes of Stb5, such as *ZWF1* and *PGI1*, could not be perturbed in the anticipated direction by overexpressing *STB5*. Indeed, Ouyang et al. also failed to pinpoint *ZWF1* – encoding the considered rate limiting enzyme of the oxidative PPP – as a target gene of Stb5 [287]. These discrepancies could potentially have to do with a more complicated regulation of these genes. Possibly, the cellular consequences brought about by oxidative stress, such as a reduced NADPH/$NADP^+$ ratio, are required for proper binding of Stb5 to its binding motif or subsequent induction.

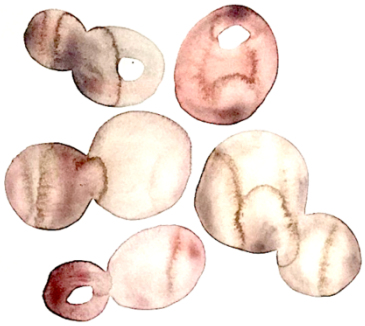
We did observe positive effects of *STB5* overexpression on FFA synthesis in the glucose phase, which was independent of the flux through the oxidative PPP (**Figure 22A** and **22B**). An increased net activity of enzymes encoded by Stb5-target genes that were activated during glucose conditions, such *ADH6* and *YMR315W*, and/or reduced consumption of NADPH via other reactions, would not have been of help during an oxidative perturbation (**Figure 21**), as it would cause an upstream blockage of the carbon flux at GAPDH, but it could still represent a source of NADPH for FA synthesis. However, the RNAseq analysis pointed out that expression of *ACC1* and *FAS1* was upregulated in response to *STB5* overexpression, which also could lead to a more efficient utilization of cytosolic acetyl-CoA. Unfortunately, the impact of these two separate effects cannot be resolved.

Previous reports indicated that the $NADP^+$/NADPH ratio is difficult to perturb in a CEN.PK background via genetic modifications. In response to overexpression of the NADPH-producing

malic enzyme in the cytosol, CEN.PK113-7D counteracted imposed changes by reducing flux through the oxidative PPP, which resulted in an unchanged NADPH/$NADP^+$ ratio [289]. Furthermore, an increased cytosolic NADPH availability mediated via a deletion of a $NADP^+$-dependent glutamate dehydrogenase gene *GDH1* was found to decrease transcription of *ZWF1*, *GND1* and *ALD6* [290]. The cellular NADPH/$NADP^+$ ratio is generally held high in order to be able to drive biosynthetic reactions, and the cytosolic ratio in CEN.PK 113-7D has been reported to be 22.0 and 15.6 during exponential phase and steady state, respectively [186]. This supports that an increase in NADPH supply would be particularly damaging in the exponential phase.

Taken together, we therefore consider it highly likely that the *STB5* overexpressing strains in our study counteracted an increased NADPH synthesis by partly overriding the Stb5-mediated regulation by other mechanisms, such as transcriptionally downregulating key components of the PPP (Figure 25C), but also by consuming more NADPH, for example via enhancing expression of *ACC1/FAS1* and elevating FA synthesis.

5 Investigating acetylation sites in Fas2 for regulatory function



5.1 Studying the function of post-translational acetylation sites

Yeast metabolism is highly regulated, in part via coordinated reprogramming of metabolism on a transcriptional level. Furthermore, regulation occurs on the protein level via post-translational modifications directly affecting enzymatic activity – a mode of regulation that has the benefit of being very fast in response to environmental changes. One group of post-translational modification that has been suggested to have a high impact on regulation of metabolism are acetylations, a topic briefly touched upon in **Chapter 2.2**.

In *S. cerevisiae,* around 4000 distinct protein acetylation sites have been identified. Compared to various species, the acetylated yeast lysines were found to have a significantly higher degree of conservation than non-acetylated lysines, and about 20% of the conserved lysines had also been confirmed to be acetylated in fruit fly and human acetylome studies [49]. Furthermore, many of the identified acetylations were located in central metabolic enzymes. For example, a large proportion of enzymes in glycolysis, gluconeogenesis, and in glycine, serine and threonine metabolism were found to be acetylated [49]. Activity of the gluconeogenetic enzyme Pck1 was already a decade ago shown to critically depend on an acetylation of its lysine residue 514, without which the enzyme fails to support growth of its host on non-fermentable carbon sources [179]. However, studies investigating a potential regulatory role of the numerous acetylation sites described in reports of the *S. cerevisiae* acetylome are currently missing.

There are several ways to evaluate if and how a specific acetylation site has impact on a protein function. A general strategy is to expose growing cells to compounds that inhibit function of deacetylases. Nicotinamide (NAM) inhibits KDACs of sirtuin type while trichostatin A (TSA) inhibits KDACs in general [291, 292]. By purifying a protein of interest and studying how the NAM/TSA treatment influenced protein acetylation levels and enzyme activity, it can be determined if the PTM has a 1) enzyme-dependent removal, 2) a regulatory role in protein function. A similar effect can be achieved by deleting genes encoding certain acetyl transferases/deacetylases (or transiently knocking out activity). A drawback of these methods is that they only allow to evaluate the function of acetylations introduced or removed via specific acetyl transferases/deacetylases, and the influence of acetylations being transferred via non-specific mechanism will be overlooked. Additionally, the treatments will affect acetylation sites on multiple proteins within the cells, making an *in vivo* evaluation of enzyme

function troublesome. Moreover, if a protein has multiple acetylation sites, it does not allow to discriminate between the effects of each individual site on protein function.

Within metabolic engineering, knowledge of specific regulatory lysine sites can be used to deregulate protein function, e.g. as in the case of the acetyl-CoA synthetase from *S. enterica* described previously [235]. In order to obtain such information, one must introduce specific point mutations into the gene sequence representing a codon change and evaluate its physiologic impact. An approach based on amber suppression technology allows to genetically encode unnatural amino acids such as acetyl-lysines, and thereby directly introducing them into the protein sequence during translation. Such an expression system has been successfully implemented in *S. cerevisiae* [293] and requires the expression of an orthogonal pair of pyrrolysyl-tRNA synthetase and $tRNA_{CUA}$ which detects the amber-codon TAG and introduces an acetyl-lysine into the growing protein chain. However, the suppression of the TAG-stop codon by $tRNA_{CUA}$ might not be complete, resulting in production of truncated proteins of interest, and possible off-target effects due to natural amber-codon usage are unavoidable.

A simpler method is to replace targeted lysine residues with alternative, native, amino acids that can be considered to mimic either the acetylated or non-acetylated state. Several reports have utilized the replacement of lysine (K) with glutamine (Q) and arginine (R) to mimic a constitutively acetylated and non-acetylated amino acid, respectively [137, 294-297]. The molecular structures of the amino acids are shown in **Figure 26**. Glutamine will similar to an acetyl-group abolish the positive charge of the non-acetylated lysine while the replacement of lysine with arginine retains a positive charge without being able to be post-translationally modified. A benefit of this system is that it should only affect the function of the protein investigated, and therefore enables to evaluate enzymatic behavior *in vivo*.

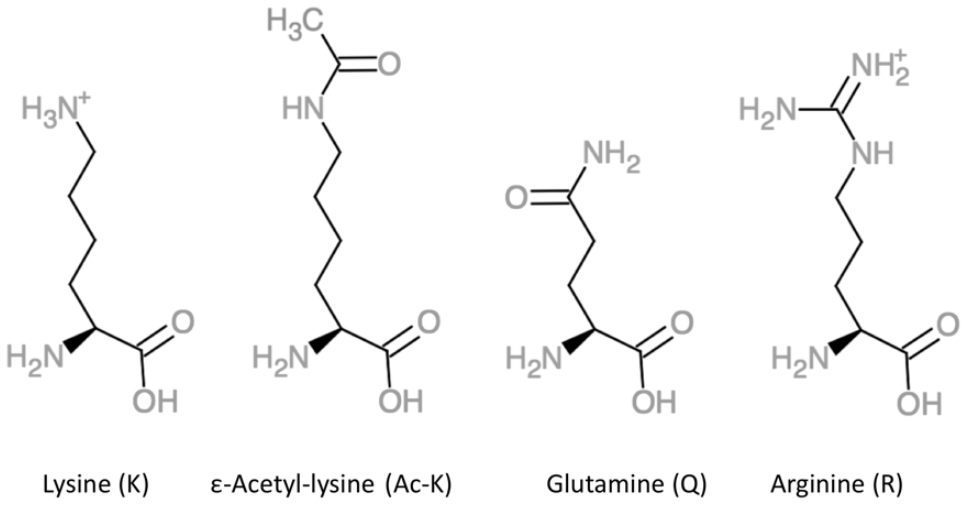


Figure 26. The structure of amino acids with relevance for the K→Q/R residue replacement system.

The residue replacement system to evaluate function of acetylations has, however, been accused of overestimating the effect of protein acetylation [298]. Furthermore, as it assumes that the lysine/acetyl-lysine functions in a protein strictly dependent on the electric charge it provides. As can be observed in Figure 26, arginine is bulkier than the unmodified lysine residue, while glutamine is smaller than the acetylated lysine. Thus, the introduction of either of the residues into the three-dimensional protein structure can interfere with function in a steric manner. Commonly, it is assumed that a particular site has a high likelihood of being of regulatory type if only one of the introduced modifications generates a difference in activity while in the other case, protein activity should remain unchanged compared to the control.

5.2 Investigation of putative regulatory acetylation of Fas2 (Paper IV)

As mentioned in **Chapter 2.3**, the study conducted by Henriksen et al. demonstrated that Acc1 as well as Fas1/Fas2 were acetylated at multiple lysine sites [49]. Based on Henriksen´s et al. data, we observed that Fas1 and Fas2 were found to have the highest number of detected unique acetylation sites in *S. cerevisiae* (29 and 50 sites, respectively). The Fas proteins are very large, but even performing a normalization to protein length, this represents an acetylation frequency of 1.41 and 2.65 per 100 amino acids in Fas1p and Fas2p, respectively. Thus, Fas1 and Fas2 belong to the top 20% and top 10% of yeast proteins with the highest frequency of acetylation, respectively. As FA biosynthesis is highly dependent on acetyl-CoA availability, whose concentration in turn influences the acetylation status of proteins, a regulatory mechanism controlling FAS activity via acetylation could be envisioned. This type of regulation of FAS activity are present in human cells, where an increased level of FAS acetylation has been correlated with an elevated degree of protein ubiquitinylation and degradation [51].

Three of the lysine acetylation sites detected by Henriksen et al. in Fas2p (K83, K173 and K1551) were confirmed in a study by Kumar et al. in which six different deletion strains (*sir2*Δ, *snf1*Δ, *snf1*Δ *sir2*Δ, *gcn5*Δ, *sir2*Δ *gcn5*Δ, *snf1*Δ *gcn5*Δ) were investigated (unpublished). K83 is located within the malonyl/palmitoyl-CoA transferase (MPT) domain located in Fas2, while the larger proportion of the MPT domain is found on Fas1 [299]. K173 is located within the acyl carrier protein (ACP) domain of Fas2, while K1551 is located within the ketoacyl synthase (KS) domain of Fas2 [38]. Due to the location of these three particular lysines within enzymatic domains of the FAS, and the fact that they were confirmed by two independent studies, they were selected to be investigated for a regulatory function by using the K→Q/R residue replacement system.

We replaced the discussed native Fas2 K-residues by introducing Q/R-codons into the *FAS2* gene sequence using the CRISPR/Cas9-technology. Conventional DNA modification strategies commonly require that a selective marker gene is introduced alongside the mutation in order to identify the correctly transformed cells. When introducing modification into central portions of an essential gene – such as *FAS2* – this interrupts the gene, thus requiring a temporal expression of the wild type gene from elsewhere during the modification process. CRISPR/Cas9 on the other hand allows to efficiently insert double-strand breaks into desired location of the gene of interest, which subsequently are repaired using the provided DNA fragments carrying the mutation to be introduced, eliminating the need for selective markers.

The cellular acetyl-CoA concentration has been shown to be the highest during exponential phase, while it decreases during the diauxic shift and in ethanol and stationary phase [139]. Thus, it is possible that post-translational regulation mediated via acetylation in certain cases is growth phase dependent, i.e. occurring in exponential phase while not in ethanol phase. Therefore, we investigated how the FAS mutants influenced FA synthesis in glucose phase as well as at stationary phase. Additionally, we cultivated the strains directly on ethanol.

Single modifications were initially introduced into the strain CEN.PK 113-7D, but the mutations did not produce a significant impact on neither growth, cellular FA content nor FA composition (data not shown). As the FAS is a highly acetylated protein, we hypothesized that the influence of a single amino acid (AA) residue on enzymatic activity might not be sufficient to determine a regulatory function. Consequently, we decided to introduce AA exchanges at all three lysine sites (K83, K173 and K1551) simultaneously to investigate the effects of the combined mutations. Furthermore, as the product of FA synthesis has multiple cellular destinations (e.g. phospholipids, triacylglycerols, acetyl-CoA via beta-oxidation), we further questioned if it would be feasible to test a regulatory function of acetylation in a strain where a small increase or reduction in FF production possibly would be masked by other counteracting

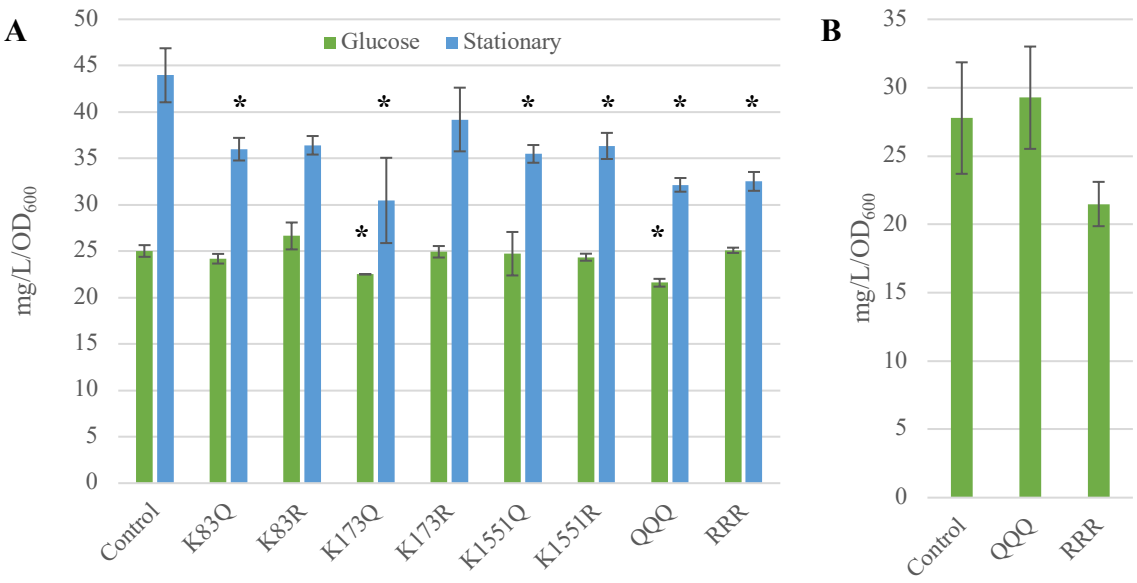


Figure 27. Total free fatty acid content (mg/L/OD_{600}) in CEN.PK113-5D *faa1*Δ *faa4*Δ strains grown in minimal medium, **(A)** containing 2% glucose harvested around glucose depletion (Glucose) or after 72 h (Stationary) and **(B)** containing 2% (v/v) ethanol harvested at 72 h. The values are means of biological triplicates ± standard deviation (except for strain K173Q, which represents duplicate measurements). Significant changes ($p<0.05$) compared to control are marked with an asterisk (*). Adapted from Bergman et al. [300].

cellular mechanisms. Therefore, we introduced the AA substitutions in the strain CEN.PK 113-5D *faa1*Δ *faa4*Δ, which is unable to reactivate FFAs into acyl-CoA, and likely experience less inhibition of FA synthesis.

When the single and triple AA substitutions were introduced into the *faa1*Δ*faa4*Δ strain, significant alterations in FFA concentrations and chain length distribution were observed. In a first experiment, the strains were cultivated in triplicates in 2% glucose (all strains) or 2% ethanol (the control and the triple mutants). For strains cultivated in glucose, samples were taken for FFA analysis at the end of the exponential phase and at 72 h, indicated as "Glucose" and "Stationary" in **Figure 27A**, respectively. These results showed that FFA production was significantly reduced ($p<0.05$) in the glucose phase in the strains which had the K173Q and triple QQQ mutations, but not in the corresponding K173R and RRR strains. At stationary phase, however, majority of the Fas2 mutants showed a significant reduction in FFA synthesis, apart from K83R ($p=0.053$) and K173R. The results for the modified K173 residue would be the expected outcome if acetylation of K173 was involved in negatively regulating FAS activity in glucose conditions. The opposite behavior was observed when the triple mutant strains were cultivated in ethanol: a (non-significant) reduction in FFA synthesis was observed exclusively for the triple RRR mutant (**Figure 27B**), which rather indicates that inability to acetylate the investigated lysine residues has a negative effect on FAS activity.

In order to obtain more reliable results, we decided to repeat the experiment using 3% glucose – resulting in a prolonged glucose phase – and cultivating the strains in quadruplicates. The triple mutants were additionally cultivated in 3% ethanol or a total of 3% glucose, where 2% was supplied via "Feed Beads", enabling a slow but stable release of glucose over time, thus corresponding to a glucose-limited cultivation. This was performed as the three lysine sites K83, K173 and K1551 had been observed by Kumar et al. during glucose-limited chemostat cultivations, and thus a potential functional impact might have been of particular importance in such conditions.

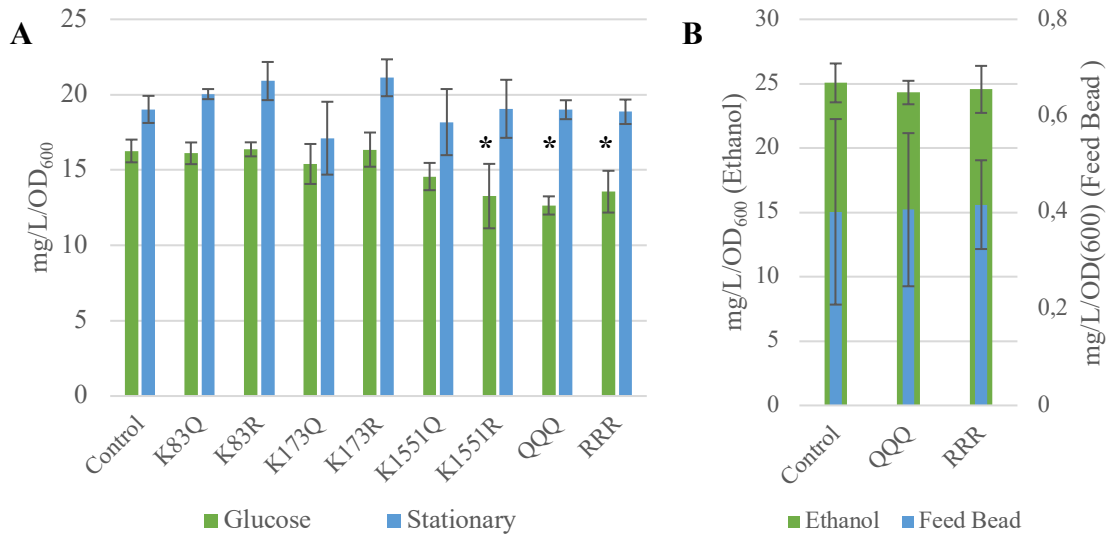


Figure 27. Total free fatty acid content (mg/L/OD_{600}) in CEN.PK113-5D *faa1*Δ *faa4*Δ strains grown in minimal medium, **(A)** containing 3% glucose harvested around glucose depletion (Glucose) or after 72 h (Stationary) and **(B)** containing 3% (v/v) ethanol harvested at 72 h (Ethanol) or 1% dissolved glucose and 2% glucose supplied in feed bead format added after 24 h of culture, harvested after 96 h (Feed Bead). The values are means of biological quadruplicates ± standard deviation (except for K173R, which represents triplicate measurements). Significant changes ($p<0.05$) marked with an asterisk (*). Adapted from Bergman et al. [300].

Figure 28 show the FFA quantification of strains cultivated in glucose (**28A**) and in ethanol or using feed beads (**28B**). The observations made in the first experiment were not reproducible in the second. In part, the significant reduction in FFA synthesis observed for strain with mutation K173Q did not manifest, while the modifications K1551R, QQQ and RRR all led to a significant reduction in FFA production at the end of the glucose phase. Furthermore, no significant effect on FFA production was observed at stationary phase, which also is contradictory to the initial results. Neither when the triple mutants were cultivated in ethanol or using feed beads, significant changes were observed compared to the control.

Intriguingly, growth under glucose-limitation (**Figure 28B**) drastically decreased FFA production, as FFA concentrations were as low as 0.4 mg/L/OD_{600} compared to 15-25 mg/L/OD_{600} when glucose or ethanol was used as substrate. Possibly this is related to a reduced lipid remodeling during glucose-limited conditions, which is believed to be the primary source of FFA species in yeast [12, 16].

Considering results from the first as well as the second experiment, both Q and R substitutions often resulted in relatively equal changes in FFA synthesis in mutant strains of K83, K1551 and the triple mutants when cultivated on glucose (see **Figure 27A** and **Figure 28A**). This indicates that for these AA substitutions, the residue replacements interfere with protein function in an "acetylation-independent manner" and are unsuitable to study the role of protein acetylation. For site K173, the Q substitution consequently had a greater negative influence on FA synthesis, even if the results were non-significant in the second experiment.

An observed difference that was consistent between the first and the second experiment was that K173Q, but not K173R appeared to influence the chain length distribution relative to the control. **Table 5** shows the percentage of C_{16} and C_{18} FFA species in the mutants cultivated in 2 and 3% glucose. Compared to the control, C_{16} species were reduced from 46% and 53% to 38% and 46% in glucose and stationary phase, respectively, when cultivated using 2% glucose. When cultivated in 3% glucose, C_{16} species decreased from 64% to 57% in glucose phase, and from 53% and 37% in stationary phase, respectively. No significant change in FA chain distribution pattern was observed for the K173R mutant cultivated in 2% glucose, while a decrease in C_{16} species was observed when cultivated in 3% glucose, albeit to a much lower

extent than its glutamine counterpart. Oddly, while 5 of the 8 Fas2 mutants displayed a significantly higher C_{16} content than the control at stationary phase when cultivated in 2% glucose, a majority of the strains exhibited a reduced C_{16} content when cultivated in 3% glucose.

Table 5. Percentages of C_{16} and C_{18} species in FFA samples taken from Fas2 mutants cultivated in either minimal medium containing 2 or 3% glucose. The samples were harvested at the end of exponential phase (Glucose) or at 72 h (Stationary). Significant changes compared to the control ($p<0.05$) are indicated in bold.

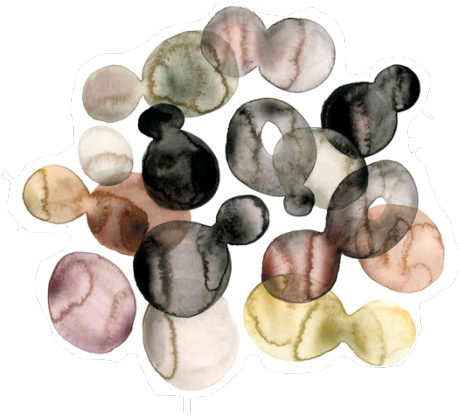
	2% glucose				**3% glucose**			
	Glucose		*Stationary*		*Glucose*		*Stationary*	
	C_{16}	**C_{18}**	**C_{16}**	**C_{18}**	**C_{16}**	**C_{18}**	**C_{16}**	**C_{18}**
Control	46±3	52±3	53±0	45±1	64±1	32±1	53±1	45±1
83Q	47±3	52±2	**57±0**	**42±0**	65±1	33±2	52±1	45±1
83R	46±1	52±1	**55±1**	**43±1**	62±2	35±2	**50±1**	**47±0**
173Q	**38±4**	**59±2**	**46±2**	**51±1**	**57±3**	**38±2**	**37±4**	**59±3**
173R	45±2	53±2	53±1	45±1	63±1	32±1	**49±1**	**49±1**
1551Q	45±4	54±4	**55±1**	**43±1**	62±3	35±3	**50±0**	**48±0**
1551R	48±2	51±3	**54±0**	**43±0**	65±1	32±1	**49±1**	**48±1**
QQQ	44±2	55±2	53±1	45±1	**59±1**	**37±2**	**47±1**	**51±1**
RRR	48±1	49±1	**54±1**	**44±1**	63±1	32±1	**48±0**	**51±0**

In the FAS, K173 is located very close to the active site of the ACP domain (Serine180). The ACP is instrumental in the FA synthesis process as it is physically attached to the growing acyl-chain via the pantetheine arm, and is responsible for the chain transfer to the different catalytic centers in the FAS complex [299]. Potentially, the introduction of glutamine at site K173 interferes with the transfer of acyl chains to the MPT domain – responsible for releasing the free acyl-CoAs. This would in part slow down FA synthesis (**Figure 27A**), as FAs would be trapped within the FAS complex, and simultaneously stimulate a release of C_{18} acyl chains at the expense of C_{16} species. In order to determine if the observed changes are a result of a regulatory acetylation on K173 or a steric alteration of the catalytic chamber when introducing a non-charged/smaller AA, further studies are however required.

In hindsight, the decision to evaluate merely three acetylated lysine sites in a protein complex reported to be able to carry at least 80 was suboptimal. Thus, a more general strategy to study protein acetylation presented in **subchapter 5.1** would potentially have been more advantageous in the case of yeast FAS, assuming that the acetylations were introduced enzymatically. Alternatively, a selection of acetylation sites known to be evolutionary conserved could have been targeted. For example, by performing sequence alignment of the *S. cerevisiae* and oleaginous yeast *Y. lipolytica* FAS, we identified 43 out of the 80 acetyl-lysine sites detected in *S. cerevisiae* FAS to be conserved between the two species, out of which 8 sites were confirmed to be acetylated in *Y. lipolytica* [131].

Yet, the degree of acetylation frequency at the particular sites is likely a stronger indicator of whether the modifications have a physiological role or not. An acetylation site is unlikely to have a regulatory impact if too few proteins carry the modification at a single time point to significantly influence its activity. Weinert et al. estimated that 95% of the acetylation sites in *S. cerevisiae* were of low-stoichiometry, defined as that <1% of the lysine-site in proteins are occupied by an acetyl-group [50]. A recent study investigating acetylation frequency in human cells showed a similar pattern, and furthermore demonstrated that the acetylation frequency of various functionally characterized sites are of low-stoichiometry *in vivo* [301]. When the data of Weinert et al. were investigated, only three acetylation sites detected within Fas2 were suggested to be of high-stoichiometry (>1%): K573, K1423 and K1551. These are therefore good candidates for a potential further analysis.

6 Conclusions and perspectives



The overall goal of this thesis was to investigate methods and system parameters with potential to improve production of or shed light on FA synthesis in the commonly utilized cell factory *S. cerevisiae*. This entailed to evaluate a more energy- and carbon-efficient route to produce the FA precursor acetyl-CoA (**Paper I** and **II**). Furthermore, we investigated if overexpression of a TF related to NADPH biosynthesis could be used as strategy to boost cofactor-expensive FA synthesis (**Paper III**). Lastly, we also sought to explore if FAS activity was regulated by acetylation – knowledge which potentially could be used to deregulate FA synthesis (**Paper IV**). It should nevertheless be highlighted that the knowledge assembled within the different projects of this thesis has potential to be used in other applications. The following subchapters will discuss the overall conclusions gained from working with the mentioned topics and put them into a more general context.

6.1 Acetyl-CoA supply via the XFPK/PTA pathway

Conclusions

When a comparison of various routes for providing cytosolic acetyl-CoA in *S. cerevisiae* is made, the XFPK/PTA pathway stands out as the one that individually provides the best theoretical yields for several production processes relying on acetyl-CoA as precursor, including FAs [43]. The theoretical yield is based on an assumption of 100% flux efficiency via the targeted pathway, and therefore does not consider aspects such as kinetic and thermodynamic constraints. In **Paper I**, we identified several efficient XFPKs with varying substrate specificity, information that can be used to optimize flux through a heterologous XFPK/PTA pathway in yeast. We later on showed that co-expression of XFPK/PTA indeed had a significant positive influence on FA synthesis in the glucose phase. However, during the course of the project, we recognized aspects of XFPK/PTA expression in *S. cerevisiae* imposing limitations on yield.

The first observation is that *S. cerevisiae* possesses a strong enzymatic ability to degrade the XFPK product AcP to acetate. Excess acetate formation was highlighted already in a study expressing the XFPK from *B. lactis* by Sonderegger et al. [245], but not discussed as constituting a problem in the more recent studies using the XFPK from *A. nidulans* [32, 45].

This makes sense when observing how limited the catalytic efficiency of the Xfpk(AN) enzyme is in comparison to that of enzymes from the *Bifidobacterium* genus. One of the major theoretical advantages of utilizing the heterologous Xfpk enzyme is its ability to bypass the ATP consumption during acetate to acetyl-CoA formation. Thus, the requirement to activate additionally formed acetate to acetyl-CoA using endogenous Acs enzymes should constitute a distinct problem when attempting to optimize acetyl-CoA supply and subsequent product formation. In **Paper II,** we showed that the excess acetate formation resulting from expression of the XFPK is likely to increase proton decoupling, leading to a decreased biomass yield and respiratory demand in batch culture. Furthermore, even in the absence of acetate accumulation in chemostat cultivation, XFPK expression led to a significant decrease in biomass yield and a concurrent increase in O_2 consumption, further emphasizing that the ATP demand is increased when acetate is converted to acetyl-CoA.

We confirmed the glycerol-3-phosphatases Gpp1 and Gpp2 to be responsible for a large proportion of the formation of acetate from AcP. However, deletion of both *GPP1* and *GPP2* increases lag time, severely decreases μ(max), and at the same time significantly increases acetate accumulation in the strains. These results verified the importance of glycerol formation in maintenance of redox homeostasis, and also showed that the purpose of preventing excessive acetate formation is defeated when deleting both *GPP1* and *GPP2*. Thus, our recommendation is that that the most influential of the two enzymes – Gpp1 – is targeted for removal when the XFPK/PTA pathway is expressed.

A second parameter that prevents an efficient flux through the XFPK/PTA pathway is related to the reversibility and/or the low flux through the PTA reaction. When a XFPK and PTA were co-expressed in a wild type strain – results of which were presented in **Chapter 3.4** – acetate accumulation was not significantly altered compared to expression of XFPK alone. Thus, the reaction to convert AcP to acetyl-CoA must have been significantly slower than the endogenous hydrolysis process to acetate. Furthermore, co-expression of XFPK/PTA resulted in a reduced final biomass yield compared to expression of XFPK alone, both in wild type and *gpp1*Δ*gpp2*Δ background. This indicates that the reversibility of the PTA reaction results in a futile cycle, where AcP is degraded to acetate via endogenous routes, ATP is consumed during acetate activation, and PTA uses acetyl-CoA to reform AcP. The net result is a consumption of ATP, that should negatively affect growth.

The thermodynamic equilibrium constraints of the PTA reaction dictate that it is likely to form AcP from acetyl-CoA when the concentration of AcP is low. Such a situation is probable to occur after glucose is depleted, and the concentration of sugar phosphates – i.e. the substrate of XPFK – decreases. Indeed, this behavior would explain the observation that even if co-expression of XFPK/PTA generated an increased ability to produce FFA in the glucose phase in a *gpp1*Δ mutant, the expression had a negative effect on the final FFA titers. In view of this, in order to ensure that the XFPK/PTA pathway promotes synthesis of FA (or other acetyl-CoA derived products), a continuous supply of AcP must be secured. This indicates that a two-stage fermentation process, which normally occurs in batch cultures, should be avoided, for example by employing a fed-batch process.

Perspectives

Taken together, in order for the XFPK/PTA pathway to reach its full potential as an efficient, acetyl-CoA providing pathway in yeast, more work is required. To decrease acetate formation further, glycerol-3-phosphatases from heterologous sources could be screened for an ability to catalyze the vital function of glycerol formation while displaying a minimal AcP-hydrolytic activity. Such candidates could be used to replace the native enzymes. Furthermore, engineering of PTA to display stronger acetyl-CoA forming behavior would be highly beneficial for the

overall pathway flux. Following such optimization work, interesting areas of XFPK/PTA utilization are highlighted in the following paragraphs.

The stoichiometric analysis performed by van Rossum et al. showed that an ultimate strategy to provide cytosolic acetyl-CoA for FA synthesis is a combinatorial approach of XFPK/PTA flux (65%) and ATP-neutral pyruvate to acetyl-CoA flux (35%) [43]. As previously mentioned, such an approach was successfully utilized by Meadows et al. to produce farnesene, resulting in an process with increased yield, productivity as well as decreased oxygen consumption [276], The study clearly demonstrates the broad applicability and benefit of the XFPK/PTA pathway in optimization of microbial catalyst for acetyl-CoA derived production. This study utilized the XFPK from *L. mesenteroides* also investigated within this thesis, which we observed had a relatively weak ability to convert F6P to AcP. Their procedure to select the best performing XFPK/PTA couple was to perform growth complementation of an *acs2*Δ mutant on glucose. This likely could have hidden more active XFPK candidates as an increased acetate-accumulation likely would have restricted the growth of such strains. In order to optimize redirection of the carbon flux when glucose is used as feedstock, we suggest to utilize an enzyme with strong affinity toward F6P (e.g. Xfpk(BB)), as F6P then is present at higher concentration than X5P.

On the other hand, candidates with a more pronounced affinity for X5P would be beneficial in order to process pentose sugars such as xylose. Pentose sugars are a common constituent of lignocellulosic material, and may constitute up to 20% of the total sugar content [302]. In order for microbial catalysts to truly constitute a climate neutral and sustainable route of chemical production, full utilization of these resources is essential. Naturally, *S. cerevisiae* does not grow on xylose, leading to a substantial resource waste when lignocellulosic material is fermented. Consequently, during the last two decades plenty of resources have been invested to engineer yeast for xylose fermentation [303]. Sonderegger et al. evaluated the use of the XFPK from *B. lactis* to promote ethanol fermentation on xylose [245]. Flux through the redox neutral XFPK/PTA-pathway relieves a redox imbalance otherwise resulting from the sequential activity of a NADH-forming heterologous xylitol dehydrogenase and GAPDH, which under normal circumstances limits the attainable ethanol yield. As the strains expressing Xfpk(BL) accumulated excess acetate – a severe inhibitor of xylose fermentation – the choice was made to rely on a weak endogenous XFPK-activity detected in *S. cerevisiae*, even though suboptimal flux through the pathway was observed [245]. Thus, the knowledge of the role of Gpp1 and Gpp2 in AcP hydrolysis should bring substantial benefit for utilizing a XFPK/PTA-pathway in ethanol fermentation of xylose. If considering FA synthesis from xylose, it would be interesting to evaluate a xylose activation strategy based on the redox neutral xylose isomerase (XI) directly converting xylose to xylulose [303], in combination with the XFPK/PTA-pathway. This would enable a redox neutral route to acetyl-CoA, which is preferential compared to a NADH-forming scenario according to the principles discussed in **Chapter 3.1**. However, ATP and reducing power would still be required to be produced via other routes.

An additional potential application of XFPK expression is related to the intracellular levels of the sugar phosphate E4P, which we in **Paper II** showed was increased more than 5-fold in the strain expressing Xfpk(BB). Aromatic chemicals are routinely produced from fossil fuels, and have large application areas within the chemical, pharmaceutical and food industries. Many metabolic engineering strategies have been designed to make their renewable synthesis more efficient [304]. Biosynthesis of the aromatic compounds proceeds via the shikimate pathway and is initiated with the formation of dehydroshikimate via condensation of the glycolytic intermediate PEP and the PPP intermediate E4P, of which E4P has been shown to be the limiting substrate [305]. Thus, expression of a XFPK candidate with high affinity for F6P resulting in formation of E4P and AcP is likely to increase formation of dehydroshikimate and metabolites derived thereof.

6.2 Increasing NADPH supply to stimulate FA synthesis

Conclusions

As a second part of this thesis, we evaluated if FA synthesis could be positively modulated by a strategy aimed to increase supply of NADPH, used in extensive amounts during FA synthesis. Increasing NADPH supply has been shown to be successful in promoting FA synthesis, in particular when redirecting flux towards the oxidative PPP. In **Paper III**, we overexpressed *STB5* – encoding a transcription factor known to regulate multiple genes related to NADPH biosynthesis in response to oxidative stress – with the hypothesis that the flux through the oxidative PPP and other NADPH-producing reactions would increase, and as a consequence stimulate FA synthesis. Even though we did observe a moderate positive effect of *STB5* overexpression on FA synthesis in the glucose phase, this effect was shown to be independent of flux through the oxidative PPP. This agreed with the observation that strains overexpressing *STB5* had a reduced ability to handle H_2O_2 stress.

Transcriptome profiling confirmed that strong *STB5* overexpression appeared to cause a counterintuitive phenotype with reduced flux through the oxidative PPP in batch phase while a larger proportion of the suggested Stb5 target genes were activated in glucose-limited conditions. Previous studies performed in CEN.PK strains has also showed that flux through the oxidative PPP or transcription of genes such as *ZWF1*, *GND1* and *ALD6* were decreased in response to genetic alterations increasing NADPH supply [289, 290]. Furthermore, the apparent inability of Stb5 to activate expression of *ZWF1* and downregulating *PGI1* the critical genes to moderate a switch from glycolytic to oxidative PPP flux – suggests that oxidative stress (in)directly is required for Stb5 to transcriptionally regulate these genes.

Overexpression of *STB5* to elevate NADPH levels is a metabolic engineering strategy with broad applicability. During the preparation of the **Paper III**, three other studies were published that investigated the use of *STB5* overexpression as means to positively influence synthesis of products depending on NADPH or a high PPP flux, some with moderate success [306-308]. None of the mentioned groups investigated which genes actually were activated in response to *STB5* overexpression and relied fully on the transcriptional regulation profile reported in the literature. Our study highlights that engineering NADPH metabolism via *STB5* overexpression has systemic effects if NADPH is not consumed efficiently.

Seen in the light of our results and those observed by others, it appears as if CEN.PK strains efficiently counteract an elevated NADPH/$NADP^+$ ratio by transcriptionally downregulating some of the most influential genes in NADPH biosynthesis. Thus, increasing NADPH supply must be tightly balanced with improving consumption in the targeted pathway in order to avoid systemic negative effects. Indeed, we observed improved growth of a strain expressing *STB5* under the weak P_{CYC1} promoter when FA synthesis was promoted in a *faa1*Δ*faa4*Δ strain.

Perspectives

In general, the optimization of metabolic engineering strategies is dependent on establishing a redox balanced formation of product. In case of FA synthesis, this would necessitate a bypass of NADH formation in glycolysis, while producing two molecules of NADPH per acetyl-CoA formed. Indeed, this could be achieved by forcing flux through the oxidative PPP and from thereon directly to acetyl-CoA synthesis, e.g. by a X5P-specific XFPK/PTA pathway.

Overexpression of *STB5* as a metabolic engineering strategy to enhance flux through the oxidative PPP – which was our intention – did not prove to be a particularly robust or effective method. In this respect, overexpression of the desired target genes by direct modulation of promoter activity [15] is likely to be more efficient. In addition, it would probably prevent a

subsequent transcriptional inactivation of the genes in response to an increased $NADPH/NADP^+$ ratio. Another alternative could be to downregulate the activity of the glycolytic gene *TPI1*, as a block of this particular reaction has been shown to force carbon flux through the oxidative PPP [309]. Such a strain would also constitute an interesting host to evaluate the XFPK/PTA pathway for enhanced acetyl-CoA supply, as reduced activity of Tpi1 also was shown to increase F6P/X5P levels.

An inherent drawback of enhancing cofactor supply is that it is an unspecific approach, as anabolic reactions in general are dependent on NADPH. Thus, both NADPH and cellular precursors will be consumed by other processes. Seen in this respect, increasing cofactor supply has the greatest potential when other parameters of production have been optimized, and NADPH supply is truly limiting. Furthermore, deleting competing non-essential enzymes consuming NADPH is an alternative, as indicated by the successful strategy to delete the $NADP^+$-dependent glutamate dehydrogenase *GDH1* for increasing fatty alcohol titers [17].

Currently, the achieved product-on-substrate yields of FA synthetic processes in *S. cerevisiae* are relatively low compared to the theoretical ones [310], which likely is why low carbon efficacy of the production host has not yet emerged as a pronounced problem. When researchers get closer the theoretical limit, an elevated flux through the oxidative PPP would however constitute a problem due to the carbon loss it is associated with. Lipid production in the oleaginous yeast *Y. lipolytica* was for example recently pushed closer to the theoretical limit by engineering an enhanced NADPH supply via non-CO_2 emitting sources by expressing heterologous, $NADP^+$-dependent versions of GAPDH and ME [311].

An ideal way to produce FAs is however through a process that does not require NADPH at all, but rather depends on NADH provided via glycolysis. This can be achieved by employing enzymes catalyzing β-oxidation in a reverse approach [312]. The process utilizes acetyl-CoA instead of malonyl-CoA for chain elongation – reducing ATP consumption – and NADH instead of NADPH as a source of electrons. Thus, this allows for a more energy and resource efficient production of FAs, and the system has been utilized in *E. coli* to efficiently produce S/MCFAs, reaching titers of up to 1-2 g/L [313, 314]. The establishment of the reversed β-oxidation in yeast has unfortunately proved to be challenging [315, 316]. Hopefully the key bottlenecks in the system can be identified to unlock this strategy also for use in *S. cerevisiae*.

6.3 Post-translational regulation of FAS

Conclusions & Perspectives

The identification of regulatory acetylation sites on enzymes poses an opportunity to deregulate connected cellular processes. Due to proximity of acetyl-CoA and FAS in the metabolic network, we hypothesized that degree of protein acetylation indeed could have a direct role in regulating enzyme activity. In **Chapter 5**, results of our investigation of three acetylation sites (K83, K173, K1551) detected within Fas2 had a potential regulatory role were presented.

The results of our study were not conclusive, partly because the results produced were not reproducible between experiments conducted, and in part because both AA substitutions appeared to influence enzyme behavior through a steric mechanism for two of the three sites. Nevertheless, the behavior of the K173 mutants, where AA changes were introduced in close proximity to the active site of the ACP-domain (S180) – consequently showed a larger negative influence on FFA synthesis for mutant K173Q (mimicking an acetylated residue) compared to K173R mutant. Furthermore, the K173Q mutation specifically generated a reproducible and significant decrease in FA species with chain length C_{16} at the expense of C_{18}. The phenotype could potentially be explained by a reduced ability of ACP to efficiently transfer acyl-CoA

species to the MPT domain. More studies have to be performed in order to determine if any of the investigated residues have a regulatory role.

A study that investigated the degree of acetylation stoichiometry in *S. cerevisiae* suggests that very few of the acetylation sites detected in FAS have an important physiological function [50]. It is estimated that only three detected acetylation sites in Fas2 (and none in Fas1) have a higher frequency of occupancy than 1% - K1551 being one of them. Thus, if further studies are performed to characterize the influence of acetylation sites in yeast FAS function, sites with high acetylation frequency and/or those which are found to be evolutionary conserved among species are likely the best candidates to proceed with.

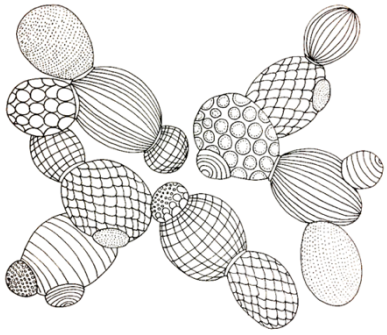
6.4 *S. cerevisiae* as a cell factory for fatty acids

The subjects explored within this thesis did not in themselves result in a *S. cerevisiae* cell factory displaying high FA yield and titers. A take-home message from the work conducted is that when providing additional amounts of acetyl-CoA or NADPH for FA synthesis, care must be taken to ensure that the produced precursors and cofactors are efficiently utilized at the point of production. Therefore, simultaneous engineering efforts to force the flux through Acc1 and the FAS complex and reduce competing pathways are required to maximize the benefit of strategies targeting an increased acetyl-CoA and/or NADPH supply to produce more FA.

Using the detailed knowledge gained within the thesis, the strategies could with little effort be implemented in platform strains already producing large quantities of FAs or lipids in order to optimize production. For example, two yeast strains were recently constructed in which ethanol formation was abolished to increase production of FAs or metabolites derived of acetyl-CoA, which potentially would benefit further from expressing the XFPK/PTA pathway [15, 249].

The progress in transforming *S. cerevisiae* to a cell factory for FAs and FA-derived chemicals has advanced tremendously just during the past five years, enabling greatly elevated product titers of various FA derived products [310]. Recent achievements in titer are 33.4 g/L FFA [15], 1.76 g/L TAGs [18]) and 6.0 g/L fatty alcohols [17]. The corresponding product-on-substrate yields for the processes were reported to be 30%, 27% and 18% of the theoretical maximum, respectively. These developments show that it is possible to transform a non-oleaginous yeast into an efficient producer of FAs, and that the production parameters likely will be pushed even further in a near future. Hopefully, the work presented in this thesis will aid in this process.

7 Acknowledgements



I am extremely grateful to have made it to this point. Repeatedly, I doubted whether I was resourceful enough to complete this journey. Now that I'm here, I have numerous people I want to acknowledge for providing me with strength, direction and support along the way.

Jens, thank you for accepting me into the Sysbio group and for providing an environment of great scientific freedom and creativity in which to freely test my hypotheses. Even though the results were not thrilling, you always remained positive throughout the process, and it allowed me to become an independent and critical researcher. Verena, you have been instrumental in this process. Thank you for always providing an open door, for the guidance in molecular biology, the detailed input (and hyphen removal) you gave on every lengthy piece of text I supplied you with, and for the empathy when I fell apart. Yun, the XFPK-project kept me motivated at times when I otherwise felt disoriented, thank you for the long discussions and guidance you gave in this particular topic of my PhD.

Rahul, without you I would not initially have entered this journey. Thank you for motivating me at the darker times, our scientific discussions and for providing me with an unofficial mentor at the initial stages of my PhD. Without your support I would likely have lost my way.

I particularly want to thank my co-authors. John, I'm so happy for your help during the last year, your willingness and ability to learn how to perform RNAseq analysis from scratch greatly improved my last two papers. Leonie, thank you for working with me in the FAS-project, even though the results were not what we hoped for, your contribution and company was much appreciated. Dóra, thank you for accepting a position as my master student, for being patient while I tried to learn how to supervise, and for continuing working hard even when I left for parental leave. Additionally, I'm thankful to Thomas and the Umeå Metabolomics Center for developing a method to quantify sugar phosphates in yeast.

Being part of the Sys(Ind)bio family was a fantastic experience. Thank you for all the inspiring research I got take part of and learn from. You made the hard times easier to live with by providing fruitful discussions and suggestions – with regards to scientific as well as personal matters – but probably more importantly, by sharing a lot fun: Bouke, Stefan, Yasaman, Leonie, Benjamin, Raphael, Paulo, Yating, Jichen, Christoph, Jens, John, Sakda, Julia, Jacob, Nina, Emma, Lina, Jenny, Amir, Rui, Martina, Marie, Angelica, Shaq, Martin, Kate, Promi, Zongjie, Yongjin, Zhiwei, Rosemary, Tao, Ievgeniia, Dina, Joakim, Christer, Xin, and many more! A special thanks goes to Anastasia, Florian, Michi, Ed, Julle, Linnea, Leif, Gatto, Petri and Kaisa

– the many events of lunches, climbing, parties, dancing, singing, hangouts, festivals, travels, and dinners we shared during the early years of my PhD I will always remember with a smile.

To all the excellent administrative and lab engineer staff: Erica, Martina, Anne-Lise, Marie, Ximena, Andreas, Angelica, Emelie and Fredrik – thank you for providing a smooth working environment and making PhD-student life easy! Special thanks to Fredrik, Rui and Ed for providing excellent technical and mental support when bioreactors were behaving weirdly or got contaminated.

Sanna, my Chalmers partner-in-crime since 2007, from the potato-coordinate system to parenting and becoming a scientist. Your friendship has meant so much to me during these intense years, thank for making me trust in myself. I'm furthermore very glad and grateful to have friends like Lena, Jossan, Sofia, Mika, Filippa, Rose, Tora, Andreas, Ida, Ellen, Per and Liisa & the "Berget-parents" who provided me with a lot of fun and support along the way!

I want to thank my supportive new colleagues and my current employer TATAA Biocenter for permitting me work part time so I was able to finish this thesis. The provision of a setting to learn new things, increasing my confidence level and keeping my head focused with non-PhD work tasks is probably what have kept me sane the last year!

This journey would never have been possible without my wonderful family. Mum and Dad, thank you for the freedom you gave me while growing up, for stimulating my interest in science by letting various popular scientific journals lying around the house, and for your unconditional love and support. To my intelligent and strong sisters: Zarah and Elin, thank you for being a source of inspiration in my child- as well as adulthood, and additional appreciation to Elin for making my thesis more pleasurable to look at and in! Tommy and Elisabeth, thank you for the encouragements you gave during these years and even proof-reading parts of this thesis, but most of all for spoiling my daughter with love and attention when my PhD-endeavors kept me from doing so.

Finally, to my two most beloved. Karl, I'm so happy that you have been by my side during this experience. Your unrestricted love, humor, wonderful food, care for Lilla I, and reassurances that I would make it to this point has meant the world to me. Ingrid, thank you for the happiness, laughter, singing, curiosity and affection you give us every day. You have been a source of inspiration from the day you arrived, keeping me focused on what is truly important. I hope this thesis one day can inspire you to achieve whatever you set your mind to. I love you.

8 References

1. Petit, J.R., et al., *Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica.* Nature, 1999. **399**(6735): p. 429-436.
2. Murray, A., K. Skene, and K. Haynes, *The circular economy: an interdisciplinary exploration of the concept and application in a global context.* Journal of Business Ethics, 2017. **140**(3): p. 369-380.
3. McCormick, K. and N. Kautto, *The bioeconomy in europe: an overview.* Sustainability, 2013. **5**(6): p. 2589-2608.
4. Pfleger, B.F., M. Gossing, and J. Nielsen, *Metabolic engineering strategies for microbial synthesis of oleochemicals.* Metabolic Engineering, 2015. **29**: p. 1-11.
5. OECD/FAO, *OECD-FAO Agricultural Outlook 2018-2027*, in *OECD-FAO Agricultural Outlook*. 2018: OECD Publishing, Paris/Food and Agriculture Organization of the United Nations, Rome.
6. Fargione, J., et al., *Land clearing and the biofuel carbon debt.* Science, 2008. **319**(5867): p. 1235-1238.
7. Guillaume, T., et al., *Carbon costs and benefits of Indonesian rainforest conversion to plantations.* Nature Communications, 2018. **9**(1):2388.
8. Isikgor, F.H. and C.R. Becer, *Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers.* Polymer Chemistry, 2015. **6**(25): p. 4497-4559.
9. Borodina, I. and J. Nielsen, *Advances in metabolic engineering of yeast* Saccharomyces cerevisiae *for production of chemicals.* Biotechnology Journal, 2014. **9**(5): p. 609-620.
10. Marella, E.R., et al., *Engineering microbial fatty acid metabolism for biofuels and biochemicals.* Current Opinion in Biotechnology, 2018. **50**: p. 39-46.
11. Chen, L., et al., *Enhancement of free fatty acid production in* Saccharomyces cerevisiae *by control of fatty acyl-CoA metabolism.* Applied Microbiology and Biotechnoly, 2014. **98**(15): p. 6739-50.
12. Leber, C., et al., *Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in* Saccharomyces cerevisiae*.* Metab Eng, 2014. **28C**: p. 54-62.
13. Runguphan, W. and J.D. Keasling, *Metabolic engineering of* Saccharomyces cerevisiae *for production of fatty acid-derived biofuels and chemicals.* Metabolic Engineering, 2014. **21**: p. 103-113.
14. Zhou, Y.J., et al., *Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories.* Nat Commun, 2016. **7**: p. 11709.
15. Yu, T., et al., *Reprogramming yeast metabolism from alcoholic fermentation to lipogenesis.* Cell, 2018. 174(6): p. 1549-1558
16. Ferreira, R., et al., *Redirection of lipid flux toward phospholipids in yeast increases fatty acid turnover and secretion.* Proceedings of the National Academy of Sciences of the United States of America, 2018. **115**(6): p. 1262-1267.
17. d'Espaux, L., et al., *Engineering high-level production of fatty alcohols by* Saccharomyces cerevisiae *from lignocellulosic feedstocks.* Metabolic Engineering, 2017. **42**: p. 115-125.
18. Ferreira, R., et al., *Metabolic engineering of* Saccharomyces cerevisiae *for overproduction of triacylglycerols.* Metabolic Engineering Communications, 2018. **6**: p. 22-27.
19. Leber, C. and N.A. Da Silva, *Engineering of* Saccharomyces cerevisiae *for the synthesis of short chain fatty acids.* Biotechnology and Bioengineering, 2014. **111**(2): p. 347-358.
20. Zhu, Z.W., et al., *Expanding the product portfolio of fungal type I fatty acid synthases.* Nature Chemical Biology, 2017. **13**(4): p. 360-362.
21. Yu, T., et al., *Metabolic engineering of* Saccharomyces cerevisiae *for production of very long chain fatty acid-derived chemicals.* Nature Communications, 2017. **8**:15587.
22. Gajewski, J., et al., *Engineering fungal de novo fatty acid synthesis for short chain fatty acid production.* Nature Communications, 2017. **8**:14650
23. Buijs, N.A., et al., *Long-chain alkane production by the yeast* Saccharomyces cerevisiae*.* Biotechnology and Bioengineering, 2014. 112(6): p. 1275-1279
24. Kang, M.K., et al., *Functional screening of aldehyde decarbonylases for long-chain alkane production by* Saccharomyces cerevisiae*.* Microbial Cell Factories, 2017. **16**(1):74.
25. Zhou, Y.J., et al., *Harnessing yeast peroxisomes for biosynthesis of fatty acid-derived biofuels and chemicals with relieved side-pathway competition.* Journal of the American Chemical Society, 2016. **138**(47): p. 15368-15377.
26. Bergenholm, D., et al., *Modulation of saturation and chain length of fatty acids in* Saccharomyces cerevisiae *for production of cocoa butter-like lipids.* Biotechnology and Bioengineering, 2018. **115**(4): p. 932-942.
27. Wei, Y.J., et al., *Expression of cocoa genes in* Saccharomyces cerevisiae *improves cocoa butter production.* Microbial Cell Factories, 2018. **17**(1):11.

28. Wei, Y.J., et al., *Increasing cocoa butter-like lipid production of* Saccharomyces cerevisiae *by expression of selected cocoa genes.* AMB Express, 2017. **7**(1):34.
29. Thompson, R.A. and C.T. Trinh, *Enhancing fatty acid ethyl ester production in* Saccharomyces Cerevisiae *through metabolic engineering and medium optimization.* Biotechnology and Bioengineering, 2014. **111**(11): p. 2200-2208.
30. Shi, S., et al., *Engineering of chromosomal wax ester synthase integrated Saccharomyces cerevisiae mutants for improved biosynthesis of fatty acid ethyl esters.* Biotechnology and Bioengineering, 2014. **111**(9): p. 1740-1747.
31. Valle-Rodriguez, J.O., et al., *Metabolic engineering of* Saccharomyces cerevisiae *for production of fatty acid ethyl esters, an advanced biofuel, by eliminating non-essential fatty acid utilization pathways.* Applied Energy, 2014. **115**: p. 226-232.
32. de Jong, B.W., et al., *Improved production of fatty acid ethyl esters in* Saccharomyces cerevisiae *through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway.* Microbial Cell Factories, 2014. **13**(1):39.
33. Eriksen, D.T., et al., *Orthogonal fatty acid biosynthetic pathway improves fatty acid ethyl ester production in* Saccharomyces cerevisiae*.* ACS Synthetic Biology, 2015. **4**(7): p 808-814.
34. Wenning, L., et al., *Establishing very long-chain fatty alcohol and wax ester biosynthesis in* Saccharomyces cerevisiae*.* Biotechnology and Bioengineering, 2017. **114**(5): p. 1025-1035.
35. Wenning, L., et al., *Increasing jojoba-like wax ester production in* Saccharomyces cerevisiae *by enhancing very long-chain, monounsaturated fatty acid synthesis.* Microbial Cell Factories, 2019. **18**(1):49.
36. Lipmann, F., et al., *Coenzyme for acetylation, a pantothenic acid derivative.* Journal of Biological Chemistry, 1947. **167**(3): p. 869-870.
37. Waite, M. and S.J. Wakil, *Studies on mechanism of fatty acid synthesis. XII. Acetyl coenzyme A carboxylase.* Journal of Biological Chemistry, 1962. **237**(9): p. 2750-2757.
38. Lomakin, I.B., Y. Xiong, and T.A. Steitz, *The crystal structure of yeast fatty acid synthase, a cellular machine with eight active sites working together.* Cell, 2007. **129**(2): p. 319-332.
39. Chirala, S.S., *Coordinated regulation and inositol-mediated and fatty acid-mediated repression of fatty-acid synthase genes in* Saccharomyces cerevisiae*.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(21): p. 10232-10236.
40. Shirra, M.K., et al., *Inhibition of acetyl coenzyme A carboxylase activity restores expression of the INO1 gene in a snf1 mutant strain of* Saccharomyces cerevisiae*.* Molecular and Cellular Biology, 2001. **21**(17): p. 5710-5722.
41. Shi, S., et al., *Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1.* MBio, 2014. **5**(3): p. e01130-14.
42. Faergeman, N.J. and J. Knudsen, *Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling.* Biochemical Journal, 1997. **323**: p. 1-12.
43. van Rossum, H.M., et al., *Engineering cytosolic acetyl-coenzyme A supply in* Saccharomyces cerevisiae*: Pathway stoichiometry, free-energy conservation and redox-cofactor balancing.* Metabolic Engineering, 2016. **36**: p. 99-115.
44. Papini, M., et al., *Physiological characterization of recombinant* Saccharomyces cerevisiae *expressing the Aspergillus nidulans phosphoketolase pathway: validation of activity through C-13-based metabolic flux analysis.* Applied Microbiology and Biotechnology, 2012. **95**(4): p. 1001-1010.
45. Kocharin, K., V. Siewers, and J. Nielsen, *Improved polyhydroxybutyrate production by* Saccharomyces cerevisiae *through the use of the phosphoketolase pathway.* Biotechnology and Bioengineering, 2013. **110**(8): p. 2216-2224.
46. Stincone, A., et al., *The return of metabolism: biochemistry and physiology of the pentose phosphate pathway.* Biological Reviews, 2015. **90**(3): p. 927-963.
47. Larochelle, M., et al., *Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production.* Molecular and Cellular Biology, 2006. **26**(17): p. 6690-6701.
48. Zhao, S., et al., *Regulation of cellular metabolism by protein lysine acetylation.* Science, 2010. **327**(5968): p. 1000-4.
49. Henriksen, P., et al., *Proteome-wide analysis of lysine acetylation suggests its broad regulatory scope in* Saccharomyces cerevisiae*.* Molecular & Cellular Proteomics, 2012. **11**(11): p. 1510-1522.
50. Weinert, B.T., et al., *Acetylation dynamics and stoichiometry in* Saccharomyces cerevisiae*.* Molecular Systems Biology, 2014. **10**(1).
51. Lin, H.-P., et al., *Destabilization of fatty acid synthase by acetylation inhibits de novo lipogenesis and tumor cell growth.* Cancer Research, 2016. **76**(23): p. 6924-6936.

52. Barnett, J.A., *A history of research on yeasts. 1: Work by chemists and biologists 1789-1850.* Yeast, 1998. **14**(16): p. 1439-51.
53. Barnett, J.A., *A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850-1880.* Yeast, 2000. **16**(8): p. 755-71.
54. Barnett, J.A. and F.W. Lichtenthaler, *A history of research on yeasts 3: Emil Fischer, Eduard Buchner and their contemporaries, 1880-1900.* Yeast, 2001. **18**(4): p. 363-88.
55. Barnett, J.A., *A history of research on yeasts 5: the fermentation pathway.* Yeast, 2003. **20**(6): p. 509-43.
56. van Maris, A.J., et al., *Alcoholic fermentation of carbon sources in biomass hydrolysates by* Saccharomyces cerevisiae*: current status.* Antonie Van Leeuwenhoek, 2006. **90**(4): p. 391-418.
57. Ostergaard, S., et al., *Physiological studies in aerobic batch cultivations of* Saccharomyces cerevisiae *strains harboring the MEL1 gene.* Biotechnology and Bioengineering, 2000. **68**(3): p. 252-259.
58. Kruckeberg, A.L., *The hexose transporter family of* Saccharomyces cerevisiae*.* Archives of Microbiology, 1996. **166**(5): p. 283-292.
59. Boles, E. and C.P. Hollenberg, *The molecular genetics of hexose transport in yeasts.* FEMS Microbiology Reviews, 1997. **21**(1): p. 85-111.
60. Lindqvist, Y., et al., *Three-dimensional structure of transketolase, a thiamine diphosphate dependent enzyme, at 2.5 A resolution.* EMBO J, 1992. **11**(7): p. 2373-2379.
61. Gunsalus, I.C., B.L. Horecker, and W.A. Wood, *Pathways of carbohydrate metabolism in microorganisms.* Bacteriological Reviews, 1955. **19**(2): p. 79-128.
62. Clasquin, M.F., et al., *Riboneogenesis in yeast.* Cell, 2011. **145**(6): p. 969-980.
63. Pronk, J.T., H. Yde Steensma, and J.P. Van Dijken, *Pyruvate metabolism in* Saccharomyces cerevisiae*.* Yeast, 1996. **12**(16): p. 1607-1633.
64. Lutstorf, U. and R. Megnet, *Multiple forms of alcohol dehydrogenase in* Saccharomyces cerevisiae*. I. Physiological control of ADH-2 and properties of ADH-2 and ADH-4.* Archievs of Biochemistry and Biophysics, 1968. **126**(3): p. 933-44.
65. van Gulik, W.M. and J.J. Heijnen, *A metabolic network stoichiometry analysis of microbial growth and product formation.* Biotechnology and Bioengineering, 1995. **48**(6): p. 681-98.
66. van Dijken, J.P. and W.A. Scheffers, *Redox balances in the metabolism of sugars by yeasts.* FEMS Microbiology Letters, 1986. **32**(3-4): p. 199-224.
67. De Deken, R.H., *The Crabtree effect: a regulatory system in yeast.* Journal of Genetics and Microbiology, 1966. **44**(2): p. 149-56.
68. Fiechter, A., G.F. Fuhrmann, and O. Kappeli, *Regulation of glucose metabolism in growing yeast cells.* Advances in Microbial Physiology, 1981. **22**: p. 123-183.
69. van Hoek, P., J.P. van Dijken, and J.T. Pronk, *Effect of specific growth rate on fermentative capacity of baker's yeast.* Applied and Environmental Microbiology, 1998. **64**(11): p. 4226-4233.
70. Paalme, T., et al., *Growth efficiency of* Saccharomyces cerevisiae *on glucose/ethanol media with a smooth change in the dilution rate (A-stat).* Enzyme and Microbial Technology, 1997. **20**(3): p. 174-181.
71. Pfeiffer, T., S. Schuster, and S. Bonhoeffer, *Cooperation and competition in the evolution of ATP-producing pathways.* Science, 2001. **292**(5516): p. 504-507.
72. Nilsson, A. and J. Nielsen, *Metabolic trade-offs in yeast are caused by F1F0-ATP synthase.* Sci Rep, 2016. **6**: p. 22264.
73. Piškur, J., et al., *How did* Saccharomyces *evolve to become a good brewer?* Trends in Genetics, 2006. **22**(4): p. 183-186.
74. Thomson, J.M., et al., *Resurrecting ancestral alcohol dehydrogenases from yeast.* Nature Genetics, 2005. **37**: p. 630.
75. Molenaar, D., et al., *Shifts in growth strategies reflect tradeoffs in cellular economics.* Mol Syst Biol, 2009. **5**: p. 323.
76. Pfeiffer, T. and A. Morley, *An evolutionary perspective on the Crabtree effect.* Front Mol Biosci, 2014. **1**:17.
77. de Alteriis, E., et al., *Revisiting the Crabtree/Warburg effect in a dynamic perspective: a fitness advantage against sugar-induced cell death.* Cell Cycle, 2018. **17**(6): p. 688-701.
78. Niebel, B., S. Leupold, and M. Heinemann, *An upper limit on Gibbs energy dissipation governs cellular metabolism.* Nature Metabolism, 2019. **1**(1): p. 125-132.
79. Krivoruchko, A., et al., *Microbial acetyl-CoA metabolism and metabolic engineering.* Metabolic Engineering, 2014. **28C**: p. 28-42.
80. Galdieri, L., et al., *Protein acetylation and acetyl coenzyme A metabolism in budding yeast.* Eukaryotic Cell, 2014. **13**(12): p. 1472-1483.

81. Bender, T., G. Pena, and J.C. Martinou, *Regulation of mitochondrial pyruvate uptake by alternative pyruvate carrier complexes.* EMBO Journal, 2015. **34**(7): p. 911-924.
82. Bowman, S.B., et al., *Positive regulation of the Lpd1 gene of* Saccharomyces cerevisiae *by the Hap2/Hap3/Hap4 activation system.* Molecular & General Genetics, 1992. **231**(2): p. 296-303.
83. Gey, U., et al., *Yeast pyruvate dehydrogenase complex is regulated by a concerted activity of two kinases and two phosphatases.* Journal of Biological Chemistry, 2008. **283**(15): p. 9759-9767.
84. Graybill, E.R., et al., *Functional comparison of citrate synthase isoforms from* S. cerevisiae*.* Archives of Biochemistry and Biophysics, 2007. **465**(1): p. 26-37.
85. Ronne, H., *Glucose repression in fungi.* Trends in Genetics, 1995. **11**(1): p. 12-17.
86. Gancedo, J.M., *Yeast carbon catabolite repression.* Microbiology and Molecular Biology Reviews, 1998. **62**(2): p. 334-361.
87. Ganzhorn, A.J., et al., *Kinetic characterization of yeast alcohol dehydrogenases. Amino acid residue 294 and substrate specificity.* Journal of Biological Chemistry, 1987. **262**(8): p. 3754-3761.
88. Meaden, P.G., et al., *The ALD6 gene of* Saccharomyces cerevisiae *encodes a cytosolic, Mg(2+)-activated acetaldehyde dehydrogenase.* Yeast, 1997. **13**(14): p. 1319-1327.
89. White, W.H., et al., *Specialization of function among aldehyde dehydrogenases: The ALD2 and ALD3 genes are required for beta-alanine biosynthesis in* Saccharomyces cerevisiae*.* Genetics, 2003. **163**(1): p. 69-77.
90. van den Berg, M.A., et al., *The two acetyl-coenzyme A synthetases of* Saccharomyces cerevisiae *differ with respect to kinetic properties and transcriptional regulation.* Journal of Biological Chemistry, 1996. **271**(46): p. 28953-28959.
91. Chen, Y., V. Siewers, and J. Nielsen, *Profiling of cytosolic and peroxisomal acetyl-CoA metabolism in* Saccharomyces cerevisiae*.* PLoS One, 2012. **7**(8):e42475.
92. Huh, W.K., et al., *Global analysis of protein localization in budding yeast.* Nature, 2003. **425**(6959): p. 686-691.
93. Kals, M., et al., *YPL.db2: the Yeast Protein Localization database, version 2.0.* Yeast, 2005. **22**(3): p. 213-218.
94. Takahashi, H., et al., *Nucleocytosolic acetyl-coenzyme A synthetase is required for histone acetylation and global transcription.* Molecular Cell, 2006. **23**(2): p. 207-217.
95. Buu, L.M., Y.C. Chen, and F.J.S. Lee, *Functional characterization and localization of acetyl-CoA hydrolase, Ach1p, in Saccharomyces cerevisiae.* Journal of Biological Chemistry, 2003. **278**(19): p. 17203-17209.
96. Lee, F.J., L.W. Lin, and J.A. Smith, *A glucose-repressible gene encodes acetyl-CoA hydrolase from* Saccharomyces cerevisiae. Journal of Biological Chemistry, 1990. **265**(13): p. 7413-7418.
97. Fleck, C.B. and M. Brock, *Re-characterisation of* Saccharomyces cerevisiae *Ach1p: fungal CoA-transferases are involved in acetic acid detoxification.* Fungal Genetics and Biology, 2009. **46**(6-7): p. 473-485.
98. van Rossum, H.M., et al., *Alternative reactions at the interface of glycolysis and citric acid cycle in* Saccharomyces cerevisiae. FEMS Yeast Research, 2016. **16**(3):fow017.
99. Chen, Y., et al., *Ach1 is involved in shuttling mitochondrial acetyl units for cytosolic C2 provision in* Saccharomyces cerevisiae *lacking pyruvate decarboxylase.* FEMS Yeast Research, 2015. **15**(3):fov015.
100. White, W.H., P.L. Gunyuzlu, and J.H. Toyn, Saccharomyces cerevisiae *is capable of de novo pantothenic acid biosynthesis involving a novel pathway of beta-alanine production from spermine.* Journal of Biological Chemistry, 2001. **276**(14): p. 10794-10800.
101. Olzhausen, J., S. Schubbe, and H.J. Schuller, *Genetic analysis of coenzyme A biosynthesis in the yeast* Saccharomyces cerevisiae*: identification of a conditional mutation in the pantothenate kinase gene CAB1.* Current Genetics, 2009. **55**(2): p. 163-173.
102. Stolz, J. and N. Sauer, *The fenpropimorph resistance gene FEN2 from* Saccharomyces cerevisiae *encodes a plasma membrane H^+-pantothenate symporter.* Journal of Biological Chemistry, 1999. **274**(26): p. 18747-18752.
103. Daugherty, M., et al., *Complete reconstitution of the human coenzyme A biosynthetic pathway via comparative genomics.* Journal of Biological Chemistry, 2002. **277**(24): p. 21431-21439.
104. Rock, C.O., et al., *Pantothenate kinase regulation of the intracellular concentration of coenzyme A.* Journal of Biological Chemistry, 2000. **275**(2): p. 1377-1383.
105. Olzhausen, J., et al., *Molecular characterization of the heteromeric coenzyme A-synthesizing protein complex (CoA-SPC) in the yeast* Saccharomyces cerevisiae*.* FEMS Yeast Research, 2013. **13**(6): p. 565-573.
106. Swiegers, J.H., et al., *Carnitine-dependent metabolic activities in* Saccharomyces cerevisiae: *three carnitine acetyltransferases are essential in a carnitine-dependent strain.* Yeast, 2001. **18**(7): p. 585-95.

107. Palmieri, L., et al., *Identification of the mitochondrial carnitine carrier in* Saccharomyces cerevisiae. Febs Letters, 1999. **462**(3): p. 472-476.
108. Elgersma, Y., et al., *Peroxisomal and mitochondrial carnitine acetyltransferases of* Saccharomyces cerevisiae *are encoded by a single gene.* EMBO Journal, 1995. **14**(14): p. 3472-3479.
109. Schmalix, W. and W. Bandlow, *The ethanol-inducible Yat1 gene from yeast encodes a presumptive mitochondrial outer carnitine acetyltransferase.* Journal of Biological Chemistry, 1993. **268**(36): p. 27428-27439.
110. van Roermund, C.W.T., et al., *The membrane of peroxisomes in* Saccharomyces Cerevisiae *is impermeable to NAD(H) and acetyl-Coa under in vivo conditions.* EMBO Journal, 1995. **14**(14): p. 3480-3486.
111. van Maris, A.J.A., et al., *Overproduction of threonine aldolase circumvents the biosynthetic role of pyruvate decarboxylase in glucose-limited chemostat cultures of* Saccharomyces cerevisiae. Applied and Environmental Microbiology, 2003. **69**(4): p. 2094-2099.
112. Duntze, W., et al., *Studies on the regulation and localization of the glyoxylate cycle enzymes in* Saccharomyces cerevisiae. European Journal of Biochemistry, 1969. **10**(1): p. 83-89.
113. Lewin, A.S., V. Hines, and G.M. Small, *Citrate synthase encoded by the Cit2 gene of* Saccharomyces cerevisiae *is peroxisomal.* Molecular and Cellular Biology, 1990. **10**(4): p. 1399-1405.
114. Regev-Rudzki, N., et al., *Yeast aconitase in two locations and two metabolic pathways: Seeing small amounts is believing.* Molecular Biology of the Cell, 2005. **16**(9): p. 4163-4171.
115. Fernandez, E., F. Moreno, and R. Rodicio, *The ICL1 gene from* Saccharomyces cerevisiae. Eur J Biochem, 1992. **204**(3): p. 983-990.
116. Taylor, K.M., et al., *Localization and targeting of isocitrate lyases in* Saccharomyces cerevisiae. Biochemical Journal, 1996. **319**(Pt 1): p. 255-62.
117. Hartig, A., et al., *Differentially regulated malate synthase genes participate in carbon and nitrogen metabolism of* S. cerevisiae. Nucleic Acids Research, 1992. **20**(21): p. 5677-5686.
118. Kunze, M., et al., *Targeting of malate synthase 1 to the peroxisomes of* Saccharomyces cerevisiae *cells depends on growth on oleic acid medium.* European Journal of Biochemistry, 2002. **269**(3): p. 915-922.
119. Minard, K.I. and L. McAlister-Henn, *Isolation, nucleotide sequence analysis, and disruption of the MDH2 gene from* Saccharomyces cerevisiae*: evidence for three isozymes of yeast malate dehydrogenase.* Molecular and Cellular Biology, 1991. **11**(1): p. 370-80.
120. Lee, Y.J., et al., *TCA cycle-independent acetate metabolism via the glyoxylate cycle in* Saccharomyces cerevisiae. Yeast, 2011. **28**(2): p. 153-166.
121. Palmieri, L., et al., *Identification of the yeast ACR1 gene product as a succinate-fumarate transporter essential for growth on ethanol or acetate.* FEBS Letters, 1997. **417**(1): p. 114-118.
122. Polakis, E.S. and W. Bartley, *Changes in the enzyme activities of* Saccharomyces cerevisiae *during aerobic growth on different carbon sources.* Biochemical Journal, 1965. **97**(1): p. 284-297.
123. Barnett, J.A. and K.D. Entian, *A history of research on yeasts - 9: regulation of sugar metabolism.* Yeast, 2005. **22**(11): p. 835-894.
124. Kayikci, O. and J. Nielsen, *Glucose repression in* Saccharomyces cerevisiae. FEMS Yeast Research, 2015. **15**(6):fov068.
125. DeVit, M.J., J.A. Waddle, and M. Johnston, *Regulated nuclear translocation of the Mig1 glucose repressor.* Molecular Biology of the Cell, 1997. **8**(8): p. 1603-1618.
126. Tachibana, C., et al., *Combined global localization analysis and transcriptome data identify genes that are directly coregulated by Adr1 and Cat8.* Molecular and Cellular Biology, 2005. **25**(6): p. 2138-2146.
127. Hiesinger, M., et al., *Contribution of Cat8 and Sip4 to the transcriptional activation of yeast gluconeogenic genes by carbon source-responsive elements.* Current Genetics, 2001. **39**(2): p. 68-76.
128. Charbon, G., et al., *Key role of Ser562/661 in Snf1-dependent regulation of Cat8p in* Saccharomyces cerevisiae *and* Kluyveromyces lactis. Molecular and Cellular Biology, 2004. **24**(10): p. 4083-4091.
129. Kouzarides, T., *Chromatin modifications and their function.* Cell, 2007. **128**(4): p. 693-705.
130. Kim, G.W. and X.J. Yang, *Comprehensive lysine acetylomes emerging from bacteria to humans.* Trends in Biochemical Sciences, 2011. **36**(4): p. 211-218.
131. Wang, G.Y., et al., *Systematic analysis of the lysine acetylome reveals diverse functions of lysine acetylation in the oleaginous yeast* Yarrowia lipolytica. AMB Express, 2017. **7**(1):94.
132. Wang, Q.J., et al., *Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux.* Science, 2010. **327**(5968): p. 1004-1007.
133. Guan, K.L. and Y. Xiong, *Regulation of intermediary metabolism by protein acetylation.* Trends in Biochemical Sciences, 2011. **36**(2): p. 108-116.
134. Starai, V.J., et al., *Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine.* Science, 2002. **298**(5602): p. 2390-2392.

135. Starai, V.J., J.G. Gardner, and J.C. Escalante-Semerena, *Residue Leu-641 of acetyl-CoA synthetase is critical for the acetylation of residue Lys-609 by the protein acetyltransferase enzyme of* Salmonella enterica*.* Journal of Biological Chemistry, 2005. **280**(28): p. 26200-26205.
136. Hallows, W.C., S. Lee, and J.M. Denu, *Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases.* Proceeding of the National Academy of Science of the United States of America, 2006. **103**(27): p. 10230-10235.
137. Schwer, B., et al., *Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2.* Proceeding of the National Academy of Science of the United States of America, 2006. **103**(27): p. 10224-9.
138. Starai, V.J., et al., *Short-chain fatty acid activation by acyl-coenzyme A synthetases requires SIR2 protein function in* Salmonella enterica *and* Saccharomyces cerevisiae*.* Genetics, 2003. **163**(2): p. 545-555.
139. Cai, L., et al., *Acetyl-CoA induces cell growth and proliferation by promoting the acetylation of histones at growth genes.* Molecular Cell, 2011. **42**(4): p. 426-437.
140. Uchida, M., et al., *Quantitative analysis of yeast internal architecture using soft X-ray tomography.* Yeast, 2011. **28**(3): p. 227-236.
141. Wagner, G.R. and R.M. Payne, *Widespread and enzyme-independent Nϵ-acetylation and Nϵ-succinylation of proteins in the chemical conditions of the mitochondrial matrix.* Journal of Biological Chemistry, 2013. **288**(40): p. 29036-29045.
142. Guan, K.L. and Y. Xiong, *Regulation of intermediary metabolism by protein acetylation.* Trends in Biochemical Sciences, 2011. **36**(2): p. 108-116.
143. Newman, J.C., W.J. He, and E. Verdin, *Mitochondrial protein acylation and intermediary metabolism: regulation by sirtuins and implications for metabolic disease.* Journal of Biological Chemistry, 2012. **287**(51): p. 42436-42443.
144. Weinert, B.T., et al., *Analysis of acetylation stoichiometry suggests that SIRT3 repairs nonenzymatic acetylation lesions.* EMBO Journal, 2015. **34**(21): p. 2620-2632.
145. Madsen, C.T., et al., *Biotin starvation causes mitochondrial protein hyperacetylation and partial rescue by the SIRT3-like deacetylase Hst4p.* Nature Communications, 2015. **6**:7726.
146. Tehlivets, O., K. Scheuringer, and S.D. Kohlwein, *Fatty acid synthesis and elongation in yeast.* Biochim Biophys Acta, 2007. **1771**(3): p. 255-270.
147. Magnuson, K., et al., *Regulation of fatty acid biosynthesis in* Escherichia coli*.* Microbiological Reviews, 1993. **57**(3): p. 522-542.
148. Hiltunen, J.K., et al., *Mitochondrial fatty acid synthesis and respiration.* Biochimica Et Biophysica Acta-Bioenergetics, 2010. **1797**(6-7): p. 1195-1202.
149. Knudsen, J., et al., *Yeast acyl-CoA-binding protein: acyl-CoA-binding affinity and effect on intracellular acyl-CoA pool size.* Biochemical Journal, 1994. **302**: p. 479-485.
150. Rasmussen, J.T., et al., *Acyl-CoA-binding protein (ACBP) can mediate intermembrane acyl-CoA transport and donate acyl-CoA for beta-oxidation and glycerolipid synthesis.* Biochemical Journal, 1994. **299**: p. 165-170.
151. Burton, M., et al., *Evolution of the acyl-CoA binding protein (ACBP).* Biochemical Journal, 2005. **392**: p. 299-307.
152. Bergman, A. and V. Siewers, *Biofuels for aviation; feedstocks, technology and implementation, Chapter 7 – "Metabolic Engineering Strategies to Convert Carbohydrates to Aviation Range Hydrocarbons"*. Editor: Christopher Chuck. 1st ed. Vol. 1. 2016, London: Elsevier.
153. Ballweg, S. and R. Ernst, *Control of membrane fluidity: the OLE pathway in focus.* Biological Chemistry, 2017. **398**(2): p. 215-228.
154. Martin, C.E., C.S. Oh, and Y.D. Jiang, *Regulation of long chain unsaturated fatty acid synthesis in yeast.* Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 2007. **1771**(3): p. 271-285.
155. Kihara, A., *Very long-chain fatty acids: elongation, physiology and related disorders.* Journal of Biochemistry, 2012. **152**(5): p. 387-395.
156. Koch, B., C. Schmidt, and G. Daum, *Storage lipids of yeasts: a survey of nonpolar lipid metabolism in Saccharomyces cerevisiae, Pichia pastoris, and Yarrowia lipolytica.* FEMS Microbiology Reviews, 2014. **38**(5): p. 892-915.
157. Merkel, O., et al., *Characterization and function in vivo of two novel phospholipases B/lysophospholipases from* Saccharomyces cerevisiae*.* Journal of Biological Chemistry, 1999. **274**(40): p. 28121-28127.
158. Athenstaedt, K. and G. Daum, *Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast* Saccharomyces cerevisiae *are localized to lipid particles.* Journal of Biological Chemistry, 2005. **280**(45): p. 37301-37309.

159. Faergeman, N.J., et al., *The acyl-CoA synthetases encoded within FAA1 and FAA4 in* Saccharomyces cerevisiae *function as components of the fatty acid transport system linking import, activation, and intracellular utilization.* Journal of Biological Chemistry, 2001. **276**(40): p. 37051-37059.
160. Scharnewski, M., et al., *Mutants of* Saccharomyces cerevisiae *deficient in acyl-CoA synthetases secrete fatty acids due to interrupted fatty acid recycling.* FEBS Journal, 2008. **275**(11): p. 2765-2778.
161. Casanovas, A., et al., *Quantitative analysis of proteome and lipidome dynamics reveals functional regulation of global lipid metabolism.* Cell Chemical Biology, 2015. **22**(3): p. 412-425.
162. Lust, G. and F. Lynen, *The inhibition of the fatty acid synthetase multienzyme complex of yeast by long-chain acyl coenzyme A compounds.* European Journal of Biochemistry, 1968. **7**(1): p. 68-72.
163. Woods, A., et al., *Yeast Snf1 is functionally related to mammalian AMP-activated protein-kinase and regulates acetyl-CoA carboxylase* in vivo*.* Journal of Biological Chemistry, 1994. **269**(30): p. 19509-19515.
164. Lopes, J.M. and S.A. Henry, *Interaction of trans and cis regulatory elements in the Ino1 promoter of* Saccharomyces cerevisiae. Nucleic Acids Research, 1991. **19**(14): p. 3987-3994.
165. Ambroziak, J. and S.A. Henry, *Ino2 and Ino4 gene products, positive regulators of phospholipid biosynthesis in* Saccharomyces cerevisiae*, form a complex that binds to the Ino1 promoter.* Journal of Biological Chemistry, 1994. **269**(21): p. 15344-15349.
166. Chirala, S.S., et al., *Analysis of FAS3/ACC regulatory region of* Saccharomyces cerevisiae*: identification of a functional UASINO and sequences responsible for fatty acid mediated repression.* Nucleic Acids Research, 1994. **22**(3): p. 412-418.
167. Schuller, H.J., et al., *Regulatory gene Ino4 of yeast phospholipid biosynthesis is positively autoregulated and functions as a transactivator of fatty acid synthase genes Fas1 and Fas2 from* Saccharomyces cerevisiae*.* Nucleic Acids Research, 1992. **20**(22): p. 5955-5961.
168. Chen, M., L.C. Hancock, and J.M. Lopes, *Transcriptional regulation of yeast phospholipid biosynthetic genes.* Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 2007. **1771**(3): p. 310-321.
169. Loewen, C.J., et al., *Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid.* Science, 2004. **304**(5677): p. 1644-1647.
170. Schuller, H.J., et al., *Importance of general regulatory factors Rap1p, Abf1p and Reb1p for the activation of yeast fatty acid synthase genes Fas1 and Fas2.* European Journal of Biochemistry, 1994. **225**(1): p. 213-222.
171. Wenz, P., et al., *A downstream regulatory element located within the coding sequence mediates autoregulated expression of the yeast fatty acid synthase gene FAS2 by the FAS1 gene product.* Nucleic Acids Research, 2001. **29**(22): p. 4625-4632.
172. Kamiryo, T., S. Parthasarathy, and S. Numa, *Evidence that acyl coenzyme A synthetase-activity is required for repression of reast acetyl coenzyme A carboxylase by exogenous fatty-acids.* Proceedings of the National Academy of Sciences of the United States of America, 1976. **73**(2): p. 386-390.
173. Ogiwara, H., et al., *Inhibition of rat-liver acetyl-Coenzyme-A carboxylase by palmitoyl-Coenzyme-A - formation of equimolar enzyme-inhibitor complex.* European Journal of Biochemistry, 1978. **89**(1): p. 33-41.
174. Rasmussen, J.T., J. Rosendal, and J. Knudsen, *Interaction of acyl-CoA-binding Protein (ACBP) on processes for which acyl-CoA is a substrate, product or inhibitor.* Biochemical Journal, 1993. **292**: p. 907-913.
175. Sumper, M. and H. Trauble, *Membranes as acceptors for palmitoyl CoA in fatty acid biosynthesis.* FEBS Letters, 1973. **30**(1): p. 29-34.
176. Feddersen, S., et al., *Transcriptional regulation of phospholipid biosynthesis is linked to fatty acid metabolism by an acyl-CoA-binding-protein-dependent mechanism in* Saccharomyces cerevisiae*.* Biochemical Journal, 2007. **407**: p. 219-230.
177. Galdieri, L. and A. Vancura, *Acetyl-CoA carboxylase regulates global histone acetylation.* Journal of Biological Chemistry, 2012. **287**(28): p. 23865-23876.
178. Jiang, R. and M. Carlson, *The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Ga183 component in the kinase complex.* Molecular and Cellular Biology, 1997. **17**(4): p. 2099-2106.
179. Lin, Y.Y., et al., *Protein Acetylation Microarray Reveals that NuA4 Controls Key Metabolic Target Regulating Gluconeogenesis.* Cell, 2009. **136**(6): p. 1073-1084.
180. Lu, J.Y., et al., *Acetylation of Yeast AMPK Controls Intrinsic Aging Independently of Caloric Restriction.* Cell, 2011. **146**(6): p. 968-978.
181. Zhang, M., L. Galdieri, and A. Vancura, *The yeast AMPK homolog SNF1 regulates acetyl coenzyme A homeostasis and histone acetylation.* Molecular and Cellular Biology, 2013. **33**(23): p. 4701-4717.

182. Bilan, D.S., et al., *C Main cellular redox couples.* Russian Journal of Bioorganic Chemistry, 2015. **41**(4): p. 341-356.
183. Bucher, T., et al., *State of oxidation-reduction and state of binding in cytosolic NADH-system as disclosed by equilibration with extracellular lactate-pyruvate in hemoglobin-free perfused rat liver.* European Journal of Biochemistry, 1972. **27**(2): p. 301-317.
184. Canelas, A.B., W.M. van Gulik, and J.J. Heijnen, *Determination of the cytosolic free NAD/NADH ratio in* Saccharomyces cerevisiae *under steady-state and highly dynamic conditions.* Biotechnology and Bioengineering, 2008. **100**(4): p. 734-743.
185. Cornish-Bowden, A., *Thermodynamic aspects of glycolysis.* Biochemical Education, 1981. **9**(4): p. 133-137.
186. Zhang, J.R., et al., *Determination of the Cytosolic NADPH/NADP Ratio in* Saccharomyces cerevisiae *using Shikimate Dehydrogenase as Sensor Reaction.* Scientific Reports, 2015. **5**:12846.
187. Lin, S.J. and L. Guarente, *Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease.* Current Opinion in Cell Biology, 2003. **15**(2): p. 241-246.
188. Bedalov, A., et al., *NAD+-dependent deacetylase Hst1p controls biosynthesis and cellular NAD+ levels in* Saccharomyces cerevisiae*.* Molecular and Cellular Biology, 2003. **23**(19): p. 7044-7054.
189. Kawai, S., et al., *Molecular cloning and identification of UTR1 of a yeast* Saccharomyces cerevisiae *as a gene encoding an NAD kinase.* FEMS Microbiology Letters, 2001. **200**(2): p. 181-184.
190. Shi, F., et al., *Identification of ATP-NADH kinase isozymes and their contribution to supply of NADP(H) in* Saccharomyces cerevisiae*.* FEBS Journal, 2005. **272**(13): p. 3337-3349.
191. Bieganowski, P., et al., *Synthetic lethal and biochemical analyses of NAD and NADH kinases in* Saccharomyces cerevisiae *establish separation of cellular functions.* Journal of Biological Chemistry, 2006. **281**(32): p. 22439-22445.
192. Strand, M.K., et al., *POS5 gene of* Saccharomyces cerevisiae *encodes a mitochondrial NADH kinase required for stability of mitochondrial DNA.* Eukaryotic Cell, 2003. **2**(4): p. 809-820.
193. Luttik, M.A.H., et al., *The* Saccharomyces cerevisiae *NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH.* Journal of Biological Chemistry, 1998. **273**(38): p. 24529-24534.
194. Bakker, B.M., et al., *The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in* Saccharomyces cerevisiae*.* Journal of Bacteriology, 2000. **182**(17): p. 4730-4737.
195. Todisco, S., et al., *Identification of the mitochondrial NAD+ transporter in* Saccharomyces cerevisiae. Journal of Biological Chemistry, 2006. **281**(3): p. 1524-1531.
196. Nogae, I. and M. Johnston, *Isolation and characterization of the ZWF1 gene of* Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase.* Gene, 1990. **96**(2): p. 161-169.
197. Juhnke, H., et al., *Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress.* Molecular and General Genetics, 1996. **252**(4): p. 456-464.
198. Gorsich, S.W., et al., *Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in* Saccharomyces cerevisiae*.* Applied Microbiology and Biotechnology, 2006. **71**(3): p. 339-349.
199. Thomas, D., H. Cherest, and Y. Surdinkerjan, *Identification of the structural gene for glucose-6-phosphate dehydrogenase in yeast - inactivation leads to a nutritional requirement for organic sulfur.* EMBO Journal, 1991. **10**(3): p. 547-553.
200. Meister, A. and M.E. Anderson, *Glutathione.* Annual Review of Biochemistry, 1983. **52**: p. 711-760.
201. Yoshimoto, A. and R. Sato, *Studies on yeast sulfite reductase. I. Purification and characterization.* Biochimica Et Biophysica Acta, 1968. **153**(3): p. 555-575.
202. Holmgren, A., *Thioredoxin and Glutaredoxin Systems.* Journal of Biological Chemistry, 1989. **264**(24): p. 13963-13966.
203. Grabowska, D. and A. Chelstowska, *The ALD6 gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity.* Journal of Biological Chemistry, 2003. **278**(16): p. 13984-13988.
204. Minard, K.I. and L. McAlister-Henn, *Sources of NADPH in yeast vary with carbon source.* Journal of Biological Chemistry, 2005. **280**(48): p. 39890-39896.
205. Miyagi, H., S. Kawai, and K. Murata, *Two sources of mitochondrial NADPH in the yeast Saccharomyces cerevisiae.* Journal of Biological Chemistry, 2009. **284**(12): p. 7553-7560.
206. Castegna, A., et al., *Identification and functional characterization of a novel mitochondrial carrier for citrate and oxoglutarate in* Saccharomyces cerevisiae*.* Journal of Biological Chemistry, 2010. **285**(23): p. 17359-17370.

207. Minard, K.I., et al., *Sources of NADPH and expression of mammalian NADP+-specific isocitrate dehydrogenases in* Saccharomyces cerevisiae*.* Journal of Biological Chemistry, 1998. **273**(47): p. 31486-31493.
208. Stephanopoulos, G. and J.J. Vallino, *Network rigidity and metabolic engineering in metabolite overproduction.* Science, 1991. **252**(5013): p. 1675-1681.
209. Gombert, A.K., et al., *Network identification and flux quantification in the central metabolism of* Saccharomyces cerevisiae *under different conditions of glucose repression.* Journal of Bacteriology, 2001. **183**(4): p. 1441-1451.
210. Llobell, A., et al., *Glutathione reductase directly mediates the stimulation of yeast glucose-6-phosphate dehydrogenase by GSSG.* Biochemical Journal, 1988. **249**(1): p. 293-296.
211. Rinnerthaler, M., et al., *Yno1p/Aim14p, a NADPH-oxidase ortholog, controls extramitochondrial reactive oxygen species generation, apoptosis, and actin cable formation in yeast.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(22): p. 8658-8663.
212. Ralser, M., et al., *Metabolic reconfiguration precedes transcriptional regulation in the antioxidant response.* Nature Biotechnology, 2009. **27**(7): p. 604-605.
213. Hector, R.E., et al., *The Saccharomyces cerevisiae YMR315W gene encodes an NADP(H)-specific oxidoreductase regulated by the transcription factor Stb5p in response to NADPH limitation.* New Biotechnology, 2009. **26**(3-4): p. 171-180.
214. Ozcengiz, G. and A.L. Demain, *Recent advances in the biosynthesis of penicillins, cephalosporins and clavams and its regulation.* Biotechnology Advances, 2013. **31**(2): p. 287-311.
215. Nielsen, J., *Metabolic engineering.* Applied Microbiology and Biotechnology, 2001. **55**(3): p. 263-283.
216. Angermayr, S.A., A.G. Rovira, and K.J. Hellingwerf, *Metabolic engineering of cyanobacteria for the synthesis of commodity products.* Trends in Biotechnology, 2015. **33**(6): p. 352-361.
217. Kricka, W., J. Fitzpatrick, and U. Bond, *Metabolic engineering of yeasts by heterologous enzyme production for degradation of cellulose and hemicellulose from biomass: a perspective.* Front Microbiol, 2014. **5**:174.
218. Villadsen, I., J. Nielsen, and G. Lidén, *Bioreaction engineering principles,* Third Edition*.* New York: Springer Science+Business Media, 2011.
219. Forster, J., et al., *Genome-scale reconstruction of the* Saccharomyces cerevisiae *metabolic network.* Genome Research, 2003. **13**(2): p. 244-253.
220. Sanchez, B.J. and J. Nielsen, *Genome scale models of yeast: towards standardized evaluation and consistent omic integration.* Integrative Biology, 2015. **7**(8): p. 846-858.
221. Hinnen, A., J.B. Hicks, and G.R. Fink, *Transformation of yeast.* Proceedings of the National Academy of Sciences of the United States of America, 1978. **75**(4): p. 1929-1933.
222. Scherer, S. and R.W. Davis, *Replacement of chromosome segments with altered DNA sequences constructed* in vitro*.* Proceedings of the National Academy of Sciences of the United States of America, 1979. **76**(10): p. 4951-4955.
223. Featherstone, C. and S.P. Jackson, *DNA double-strand break repair.* Current Biology, 1999. **9**(20): p. R759-R761.
224. David, F. and V. Siewers, *Advances in yeast genome engineering.* FEMS Yeast Research, 2015. **15**(1): p 1-14.
225. DiCarlo, J.E., et al., *Genome engineering in* Saccharomyces cerevisiae *using CRISPR-Cas systems.* Nucleic Acids Research, 2013. **41**(7): p. 4336-4343.
226. Jakounas, T., et al., *Multiplex metabolic pathway engineering using CRISPR/Cas9 in* Saccharomyces cerevisiae*.* Metabolic Engineering, 2015. **28**: p. 213-222.
227. Chung, B.K.S. and D.Y. Lee, *Computational codon optimization of synthetic gene for protein expression.* BMC Systems Biology, 2012. **6**:134.
228. Hamilton, R., C.K. Watanabe, and H.A. de Boer, *Compilation and comparison of the sequence context around the AUG startcodons in* Saccharomyces cerevisiae *mRNAs.* Nucleic Acids Research, 1987. **15**(8): p. 3581-93.
229. Lee, J.W., et al., *Systems metabolic engineering of microorganisms for natural and non-natural chemicals.* Nature Chemical Biology, 2012. **8**(6): p. 536-546.
230. Eliasson, A., et al., *Anaerobic xylose fermentation by recombinant* Saccharomyces cerevisiae *carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures.* Applied and Environmental Microbiology, 2000. **66**(8): p. 3381-3386.
231. Shiba, Y., et al., *Engineering of the pyruvate dehydrogenase bypass in* Saccharomyces cerevisiae *for high-level production of isoprenoids.* Metabolic Engineering, 2007. **9**(2): p. 160-168.
232. Kozak, B.U., et al., *Replacement of the* Saccharomyces cerevisiae *acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis.* Metabolic Engineering, 2014. **21**: p. 46-59.

233. Ehsani, M., et al., *Reversal of coenzyme specificity of 2,3-butanediol dehydrogenase from* Saccharomyces cerevisae *and in vivo functional analysis.* Biotechnology and Bioengineering, 2009. **104**(2): p. 381-389.
234. Fletcher, E., et al., *Evolutionary engineering reveals divergent paths when yeast is adapted to different acidic environments.* Metabolic Engineering, 2017. **39**: p. 19-28.
235. Shiba, Y., et al., *Engineering of the pyruvate dehydrogenase bypass in* Saccharomyces cerevisiae *for high-level production of isoprenoids.* Metabolic Engineering, 2007. **9**(2): p. 160-168.
236. Chen, Y., et al., *Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism.* Metabolic Engineering, 2013. **15**: p. 48-54.
237. Boulton, C.A. and C. Ratledge, *Correlation of lipid-Accumulation in yeasts with possession of ATP:Citrate Lyase.* Journal of General Microbiology, 1981. **127**: p. 169-176.
238. Tang, X.L., H.X. Feng, and W.N. Chen, *Metabolic engineering for enhanced fatty acids synthesis in* Saccharomyces cerevisiae*.* Metabolic Engineering, 2013. **16**: p. 95-102.
239. Lian, J., et al., *Design and construction of acetyl-CoA overproducing* Saccharomyces cerevisiae *strains.* Metab Eng, 2014. **24**: p. 139-49.
240. Rodriguez, S., et al., *ATP citrate lyase mediated cytosolic acetyl-CoA biosynthesis increases mevalonate production in* Saccharomyces cerevisiae*.* Microbial Cell Factories, 2016. **15**.
241. Zhou, Y.J.J., et al., *Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories.* Nature Communications, 2016. **7**:11709.
242. Kozak, B.U., et al., *Engineering acetyl coenzyme A supply: functional expression of a bacterial pyruvate dehydrogenase complex in the cytosol of* Saccharomyces cerevisiae*.* MBio, 2014. **5**(5): p. e01696-14.
243. van Rossum, H.M., et al., *Requirements for carnitine shuttle-mediated translocation of mitochondrial acetyl moieties to the yeast cytosol.* MBio, 2016. **7**(3):e00520-16.
244. Franken, J., et al., *Reconstruction of the carnitine biosynthesis pathway from Neurospora crassa in the yeast* Saccharomyces cerevisiae*.* Applied Microbiology and Biotechnology, 2015. **99**(15): p. 6377-6389.
245. Sonderegger, M., M. Schumperli, and U. Sauer, *Metabolic engineering of a phosphoketolase pathway for pentose catabolism in* Saccharomyces cerevisiae. Applied and Environmental Microbiology, 2004. **70**(5): p. 2892-2897.
246. Medina, V.G., et al., *Elimination of glycerol production in anaerobic cultures of a* Saccharomyces cerevisiae *strain engineered to use acetic acid as an electron acceptor.* Applied and Environmental Microbiology, 2010. **76**(1): p. 190-195.
247. Waks, Z. and P.A. Silver, *Engineering a synthetic dual-organism system for hydrogen production.* Applied and Environmental Microbiology, 2009. **75**(7): p. 1867-1875.
248. Zhang, Y.M., et al., *Functional pyruvate formate lyase pathway expressed with two different electron donors in* Saccharomyces cerevisiae *at aerobic growth.* FEMS Yeast Research, 2015. **15**(4):fov024.
249. Dai, Z.J., et al., *Global rewiring of cellular metabolism renders* Saccharomyces cerevisiae *Crabtree negative.* Nature Communications, 2018. **9**(1):3059.
250. Kandler, O., *Carbohydrate metabolism in lactic acid bacteria.* Antonie Van Leeuwenhoek Journal of Microbiology, 1983. **49**(3): p. 209-224.
251. Felis, G.E. and F. Dellaglio, *Taxonomy of* Lactobacilli *and* Bifidobacteria*.* Current Issues Intestinal Microbiology, 2007. **8**(2): p. 44-61.
252. Flamholz, A., et al., *eQuilibrator-the biochemical thermodynamics calculator.* Nucleic Acids Research, 2012. **40**(D1): p. D770-D775.
253. Bogorad, I.W., T.S. Lin, and J.C. Liao, *Synthetic non-oxidative glycolysis enables complete carbon conservation.* Nature, 2013. **502**(7473): p. 693-697.
254. Cipollina, C., et al., *A comprehensive method for the quantification of the non-oxidative pentose phosphate pathway intermediates in* Saccharomyces cerevisiae *by GC-IDMS.* Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 2009. **877**(27): p. 3231-3236.
255. Suzuki, R., et al., *Overexpression, crystallization and preliminary X-ray analysis of xylulose-5-phosphate/fructose-6-phosphate phosphoketolase from* Bifidobacterium breve*.* Acta Crystallographica Section F-Structural Biology and Crystallization Communications, 2010. **66**: p. 941-943.
256. Lee, J.M., et al., *Cloning and characterization of the gene encoding phosphoketolase in* Leuconostoc mesenteroides *isolated from kimchi.* Biotechnology Letters, 2005. **27**(12): p. 853-858.
257. Liu, L.X., et al., *Phosphoketolase pathway for xylose catabolism in* Clostridium acetobutylicum *revealed by C-13 metabolic flux analysis.* Journal of Bacteriology, 2012. **194**(19): p. 5413-5422.

258. Jeong, D.W., J.M. Lee, and H.J. Lee, *Cloning and characterization of a gene encoding phosphoketolase in a* Lactobacillus paraplantarum *isolated from Kimchi.* Journal of Microbiology and Biotechnology, 2007. **17**(5): p. 822-829.
259. Yevenes, A. and P.A. Frey, *Cloning, expression, purification, cofactor requirements, and steady state kinetics of phosphoketolase-2 from* Lactobacillus plantarum*.* Bioorganic Chemistry, 2008. **36**(3): p. 121-127.
260. Meile, L., et al., *Characterization of the D-xylulose 5-phosphate/D-Fructose 6-phosphate phosphoketolase gene (xfp) from* Bifidobacterium lactis*.* Journal of Bacteriology, 2001. **183**(9): p. 2929-2936.
261. van Dijken, J.P., et al., *An interlaboratory comparison of physiological and genetic properties of four* Saccharomyces cerevisiae *strains.* Enzyme and Microbial Technology, 2000. **26**(9-10): p. 706-714.
262. Partow, S., et al., *Characterization of different promoters for designing a new expression vector in* Saccharomyces cerevisiae*.* Yeast, 2010. **27**(11): p. 955-964.
263. Racker, E., *Fructose-6-phosphate phosphoketolase from Acetobacter xylinum: F-6-P+Pi→Acetyl-P+E-4-P+H2O*, in *Methods in Enzymology*. 1962, Academic Press. p. 276-280.
264. Bergman, A., et al., *Functional expression and evaluation of heterologous phosphoketolases in Saccharomyces cerevisiae.* AMB Express, 2016. **6**(1):115.
265. Hawkins, K.M., et al., *Use of phosphoketolase and phosphotransacetylase for production of acetyl-coenzyme a derived compounds*. 2014, Google Patents.
266. Weinert, B.T., et al., *Acetyl-phosphate is a critical determinant of lysine acetylation in* E. coli. Molecular Cell, 2013. **51**(2): p. 265-272.
267. Bergman, A., et al., *Heterologous phosphoketolase expression redirects flux towards acetate, perturbs sugar phosphate pools and increases respiratory demand in Saccharomyces cerevisiae.* Microbial Cell Factories, 2019. **18**(1):25.
268. Lindberg, L., et al., *Lipidomic profiling of* Saccharomyces cerevisiae *and* Zygosaccharomyces bailii *reveals critical changes in lipid composition in response to acetic acid stress.* PLoS One, 2013. **8**(9):e73936.
269. Daran-Lapujade, P., et al., *Role of transcriptional regulation in controlling fluxes in central carbon metabolism of* Saccharomyces cerevisiae - *A chemostat culture study.* Journal of Biological Chemistry, 2004. **279**(10): p. 9125-9138.
270. van Roermund, C.W.T., et al., *Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in* Saccharomyces cerevisiae *and identification of a plasma membrane carnitine transporter, Agp2p.* EMBO Journal, 1999. **18**(21): p. 5843-5852.
271. Klotzsch, H., *Enzymatic assay of phosphotransacetylase (EC 2.3.1.8)*, in *Methods in Enzymology*. 1969. p. 381-386.
272. Campos-Bermudez, V.A., et al., *Functional dissection of* Escherichia coli *phosphotransacetylase structural domains and analysis of key compounds involved in activity regulation.* FEBS Journal, 2010. **277**(8): p. 1957-1966.
273. Andre, L., A. Hemming, and L. Adler, *Osmoregulation in* Saccharomyces cerevisiae - *studies on the osmotic induction of glycerol production and glycerol-3-phosphate dehydrogenase (NAD+).* Febs Letters, 1991. **286**(1-2): p. 13-17.
274. Pahlman, A.K., et al., *The yeast glycerol 3-phosphatases gpp1p and gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress.* Journal of Biological Chemistry, 2001. **276**(5): p. 3555-3563.
275. Nissen, T.L., et al., *Anaerobic and aerobic batch cultivations of* Saccharomyces cerevisiae *mutants impaired in glycerol synthesis.* Yeast, 2000. **16**(5): p. 463-474.
276. Meadows, A.L., et al., *Rewriting yeast central carbon metabolism for industrial isoprenoid production.* Nature, 2016. **537**(7622): p. 694-697.
277. de Jong, B.W., et al., *Metabolic pathway engineering for fatty acid ethyl ester production in* Saccharomyces cerevisiae *using stable chromosomal integration.* Journal of Industrial Microbiology and Biotechnology, 2014. **42**(3):477-86
278. Li, X.W., et al., *Overproduction of fatty acids in engineered* saccharomyces cerevisiae*.* Biotechnology and Bioengineering, 2014. **111**(9): p. 1841-1852.
279. Feng, X.Y., J.Z. Lian, and H.M. Zhao, *Metabolic engineering of* Saccharomyces cerevisiae *to improve 1-hexadecanol production.* Metabolic Engineering, 2015. **27**: p. 10-19.
280. Sandager, L., et al., *Storage lipid synthesis is non-essential in yeast.* Journal of Biological Chemistry, 2002. **277**(8): p. 6478-6482.
281. Hiltunen, J.K., et al., *The biochemistry of peroxisomal beta-oxidation in the yeast* Saccharomyces cerevisiae*.* FEMS Microbiology Reviews, 2003. **27**(1): p. 35-64.

282. Ralser, M., et al., *Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress.* J Biol, 2007. **6**(4):10.
283. Cadiere, A., V. Galeote, and S. Dequin, *The* Saccharomyces cerevisiae *zinc factor protein Stb5p is required as a basal regulator of the pentose phosphate pathway.* FEMS Yeast Research, 2010. **10**(7): p. 819-827.
284. Bergman, A., et al., *Effects of overexpression of STB5 in* Saccharomyces cerevisiae *on fatty acid biosynthesis, physiology and transcriptome.* FEMS Yeast Research, 2019. **19**(3):foz027.
285. Wasylenko, T.M., W.S. Ahn, and G. Stephanopoulos, *The oxidative pentose phosphate pathway is the primary source of NADPH for lipid overproduction from glucose in* Yarrowia lipolytica. Metabolic Engineering, 2015. **30**: p. 27-39.
286. Regenberg, B., et al., *Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in* Saccharomyces cerevisiae. Genome Biology, 2006. **7**(11):R107.
287. Ouyang, L., et al., *Integrated analysis of the yeast NADPH-regulator Stb5 reveals distinct differences in NADPH requirements and regulation in different states of yeast metabolism.* FEMS Yeast Res, 2018. **18**(8):foy091.
288. Uemura, H. and D.G. Fraenkel, *Glucose metabolism in gcr mutants of* Saccharomyces cerevisiae. Journal of Bacteriology, 2000. **182**(8): p. 2354-2354.
289. Moreira dos Santos, M., et al., *Manipulation of malic enzyme in* Saccharomyces cerevisiae *for increasing NADPH production capacity aerobically in different cellular compartments.* Metabolic Engineering, 2004. **6**(4): p. 352-363.
290. Bro, C., B. Regenberg, and J. Nielsen, *Genome-wide transcriptional response of a* Saccharomyces cerevisiae *strain with an altered redox metabolism.* Biotechnology and Bioengineering, 2004. **85**(3): p. 269-276.
291. Avalos, J.L., K.M. Bever, and C. Wolberger, *Mechanism of sirtuin inhibition by nicotinamide: Altering the NAD(+) cosubstrate specificity of a Sir2 enzyme.* Molecular Cell, 2005. **17**(6): p. 855-868.
292. Vigushin, D.M., et al., *Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo.* Clinical Cancer Research, 2001. **7**(4): p. 971-976.
293. Hancock, S.M., et al., *Expanding the genetic code of yeast for incorporation of diverse unnatural amino acids via a pyrrolysyl-tRNA synthetase/tRNA pair.* Journal of the American Chemical Society, 2010. **132**(42): p. 14819-14824.
294. Megee, P.C., et al., *Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation.* Science, 1990. **247**(4944): p. 841-845.
295. Kim, S.C., et al., *Substrate and functional diversity of lysine acetylation revealed by a proteomics survey.* Molecular Cell, 2006. **23**(4): p. 607-618.
296. Lin, R.T., et al., *Acetylation stabilizes ATP-citrate lyase to promote lipid biosynthesis and tumor growth.* Molecular Cell, 2013. **51**(4): p. 506-518.
297. Wang, Y.P., et al., *Regulation of G6PD acetylation by SIRT2 and KAT9 modulates NADPH homeostasis and cell survival during oxidative stress.* EMBO Journal, 2014. **33**(12): p. 1304-1320.
298. Fujimoto, H., et al., *A possible overestimation of the effect of acetylation on lysine residues in KQ mutant analysis.* Journal of Computational Chemistry, 2012. **33**(3): p. 239-246.
299. Jenni, S., et al., *Structure of fungal fatty acid synthase and implications for iterative substrate shuttling.* Science, 2007. **316**(5822): p. 254-261.
300. Bergman, A., et al., *Investigation of putative regulatory acetylation sites in Fas2p of Saccharomyces cerevisiae.* bioRxiv, 2018. **https://doi.org/10.1101/430918**.
301. Hansen, B.K., et al., *Analysis of human acetylation stoichiometry defines mechanistic constraints on protein regulation.* Nature Communications, 2019. **10**(1):1055.
302. Hahn-Hägerdal, B., et al., *Bio-ethanol – the fuel of tomorrow from the residues of today.* Trends in Biotechnology, 2006. **24**(12): p. 549-556.
303. Moyses, D.N., et al., *Xylose fermentation by* Saccharomyces cerevisiae*: challenges and prospects.* International Journal of Molecular Sciences, 2016. **17**(3):207.
304. Averesch, N.J.H. and J.O. Kromer, *Metabolic engineering of the shikimate pathway for production of aromatics and derived compounds - present and future strain construction strategies.* Frontiers of Bioengineering and Biotechnology, 2018. **6**:32.
305. Suastegui, M., et al., *Investigating strain dependency in the production of aromatic compounds in* Saccharomyces cerevisiae. Biotechnology and Bioengineering, 2016. **113**(12): p. 2676-2685.
306. Hong, J., et al., *Efficient production of lycopene in* Saccharomyces cerevisiae *by enzyme engineering and increasing membrane flexibility and NAPDH production.* Applied Microbiology and Biotechnology, 2019. **103**(1): p. 211-223.
307. Kim, J.E., et al., *Rerouting of NADPH synthetic pathways for increased protopanaxadiol production in* Saccharomyces cerevisiae. Scientific Reports, 2018. **8**(1):15820.

308. Park, S.H., et al., *Metabolic engineering of* Saccharomyces cerevisiae *for production of shinorine, a sunscreen material, from xylose.* ACS Synthetic Biology, 2018. **8**(2):346-357
309. Gruning, N.M., et al., *Pyruvate kinase triggers a metabolic feedback loop that controls redox metabolism in respiring cells.* Cell Metabolism, 2011. **14**(3): p. 415-427.
310. Fernandez-Moya, R. and N.A. Da Silva, *Engineering* Saccharomyces cerevisiae *for high-level synthesis of fatty acids and derived products.* FEMS Yeast Research, 2017. **17**(7):fox071.
311. Qiao, K.J., et al., *Lipid production in* Yarrowia lipolytica *is maximized by engineering cytosolic redox metabolism.* Nature Biotechnology, 2017. **35**(2): p. 173-177.
312. Dellomonaco, C., et al., *Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals.* Nature, 2011. **476**(7360): p. 355-359.
313. Kim, S., J.M. Clomburg, and R. Gonzalez, *Synthesis of medium-chain length (C6-C10) fuels and chemicals via beta-oxidation reversal in* Escherichia coli. Journal of Industrial Microbiology and Biotechnology, 2015. **42**(3): p. 465-475.
314. Kim, S. and R. Gonzalez, *Selective production of decanoic acid from iterative reversal of beta-oxidation pathway.* Biotechnology and Bioengineering, 2018. **115**(5): p. 1311-1320.
315. Lian, J. and H. Zhao, *Reversal of the beta-Oxidation Cycle in* Saccharomyces cerevisiae *for Production of Fuels and Chemicals.* ACS Synthetic Biology, 2014. **4**(3): p 332-341.
316. Teixeira, P.G., Siewers, V. and Nielsen, J., *Quantitative in vivo evaluation of the reverse β-oxidation pathway for fatty acid production in* Saccharomyces cerevisiae. bioRxiv, 2017. **http://dx.doi.org/10.1101/201616**.